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## Australia

*Patents Act 1990*

IN THE MATTER OF Australian  
Patent Application Serial No. 696764 by  
Human Genome Sciences, Inc

and

IN THE MATTER OF Opposition  
thereto by Ludwig Institute for Cancer  
Research.

### STATUTORY DECLARATION

I, Peter Adrian Walton Rogers of the Department of Obstetrics and Gynaecology, Monash University, Clayton, Victoria, Australia do solemnly and sincerely declare as follows:

#### INTRODUCTION

- 1.1 I have been asked by the Ludwig Institute for Cancer Research ("Ludwig Institute") to serve as a scientific expert in connection with Ludwig Institute's opposition to the issuance of a patent to Human Genome Sciences, Inc., ("HGS") based on HGS's Australian Patent Application No. 696764. The patent application relates generally to a gene and protein for an alleged novel vascular endothelial growth factor called "Vascular Endothelial Growth Factor 2" ("VEGF2"), and thus pertains to an area of biology closely related to my research and expertise. I understand that Ludwig Institute is a named co-applicant for a different patent application directed to subject matter that may be related to "VEGF2."
- 1.2 In February 2000 I executed a first statutory declaration to provide evidence in support of Ludwig Institute's opposition, hereinafter referred to as "OPR1" (Opponents, Peter Rogers, 1<sup>st</sup> Declaration). That first declaration included a brief summary of my scientific credentials and an introduction in which I set forth some



conventional terminology that I used throughout the declaration. I shall continue to use such terminology herein.

1.3 I hereby reaffirm my understanding that I have an overriding duty to the Patent Office (and to any Australian Federal Court that should review the Patent Office decision) to provide objective scientific analysis that I believe to be truthful. I hereby affirm that, to the best of my knowledge and belief, factual statements herein are true and opinion statements herein represent my objective scientific opinion and analysis.

1.4 My original declaration included evidence relevant to whether the opposed application lacked novelty (OPR1 at 2.1-2.8); lacked inventive step (OPR1 at 3.1-3.8); lacked sufficiency (OPR1 at 4.1-4.14); lacked fair basis (OPR1 at 5.1-5.12); lacked clarity (OPR1 at 6.1-6.11); and was not a manner of manufacture (OPR1 at 7.1-7.6). In response, the patent applicant HGS filed declarations from six scientists, John Stanley Mattick (hereinafter "AJM1" (Applicants, John Mattick, 1<sup>st</sup> Declaration)), Jennifer Ruth Gamble ("AJG1"), Nicholas Kim Hayward ("ANH1"), Thomas Rapoport ("ATR1"), Stuart Aaronsson ("ASA1"), and Susan Power ("ASP1"). In this declaration I respond to issues raised by the HGS declarants.

1.5 For the most part, the six HGS declarations are not organized by grounds of opposition, and it is not always possible to determine the issues to which the HGS declarants are responding. The HGS declarations also are repetitive of each other. I have tried to determine the issues to which HGS's evidence pertains, and reply to those issues in a topical fashion, rather than addressing each declaration serially. I have attempted to organize many of my comments in the same manner as my original OPR1 declaration, where it appears that HGS's evidence is addressing particular patent issues (e.g., novelty-inventive step or lack of fair basis/insufficiency). I have also addressed particular issues raised by individual declarants. Failure to address any specific issues should not be interpreted as agreement with any HGS declarant.

- 1.6 Unless I specifically state otherwise below, I affirm the facts and opinions expressed in my prior declaration. Nothing in HGS's evidence-in-answer causes me to change the opinions embodied in my first declaration.

#### PRELIMINARY REMARKS REGARDING QUALITY OF HGS EVIDENCE

- 2.1 I would first like to make the following preliminary observations about the HGS evidence-in-answer which are relevant to most or all patentability issues.
- A. THE HGS DECLARATIONS APPLY A SCIENTIFIC "DOUBLE STANDARD"
- 2.2 I have reviewed all of the declarations filed by HGS and observe that the declarants that reviewed Professor Alitalo's declaration (filed as part of Ludwig Institute's evidence-in-opposition) seem to believe that no conclusions can be drawn from a scientific experiment unless the experiment contains absolutely perfect, parallel positive and negative controls. (See AJG1 at 7.43 and 7.52; ANH1 at 5.6-5.13; ASA1 at 11, 13, 14, and 18] The Alitalo declaration provided direct evidence that VEGF2 cannot be expressed and secreted in the manner taught by HGS in the opposed application, but the HGS declarants suggest that such experiments are flawed and cannot form the basis for any valid conclusions.
- 2.3 The HGS declarants also are very restrictive of the conclusions that they are willing to draw based upon prior art. For example, when discussing the prior art, Dr. Mattick does not believe that any conclusions can be drawn about whether antibodies raised against a prior art VEGF polypeptide will cross react with *identical* sequences that occur in VEGF2 on a theoretical level - he would need actual experimental data. (AJM1 4.33) This is Dr. Mattick's position even though he asserts a few pages later (when commenting on sufficiency of disclosure), that "computer programs were *readily available* in 1994 to generate" information about "all of the antigenic sites on the VEGF-2 molecule." (AJM1 4.83). His opinions on these antibody issues are inconsistent.

2.4 In stark contrast, the declarants that HGS asked to evaluate the quality of the teachings of the opposed application have taken a very lenient approach. These declarants appear to have accepted that VEGF2 can be expressed in the manner suggested in the opposed application,<sup>1</sup> even though there is not a single experimental example in the opposed application in which the inventors report successful expression of VEGF2 in a cell. (See OPR1 at 3.4.2, 3.5, 4.6.2.2, and 7.6) The HGS declarants have also accepted that VEGF2 has certain biological activities, even though the opposed application contains no evidence whatsoever of biological activity; and the declarants do not appear to have performed any biological activity testing of their own. (OPR1 at 2.3.2, 2.7.4, 3.4.2, 4.6, 4.6.1-4.6.5, 4.8, 5.4, 5.5, and 5.9) These declarants have also accepted that VEGF2 can be used to diagnose or treat a wide variety of diseases, even though the opposed application contains no evidence whatsoever that VEGF2 can be used for such purposes. (OPR1 at 2.3.3-2.3.4, 3.7.1, 4.6.3, and 6.8.1-6.8.4.)

2.5 In my opinion, these two extreme approaches are irreconcilable. The experiments performed by Dr. Alitalo and described in his first declaration are more relevant to the adequacy of the teachings in the opposed application than the experiments (or lack thereof) described in the application itself, or the experiments by Dr. Power (discussed below in greater detail), which are not based on the teachings in the opposed application. (And, had HGS raised any legitimate criticisms of Dr. Alitalo's first declaration, the criticisms have been fully addressed by the experiments Dr.

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<sup>1</sup> See, e.g., AJM1 at 4.19-4.23 (Mattick cataloging uses for VEGF2 alleged in the opposed application) and 4.24 (Mattick concluding that these unsupported uses "constitutes the basic information that I would have required in 1994 to use VEGF-2 in a wide range of biological activities.") [See AJM1 at 3.31, 3.33, 4.3-4.13, 4.77-4.78; AJG1 at 6.5, 6.8, 6.11, 7.24, 7.46, 7.48; ANH1 at 3.15, 3.19-3.23, 3.26, 4.20; ATR1 at 9-12; ASA1 at 6 and 17]

Alitalo describes in his second declaration.)<sup>2</sup> When all of the scientific evidence in the opposition proceeding is viewed objectively, one finds direct evidence (from Dr. Alitalo, two sets of experiments) that the VEGF2 invention does not work as it was described in the opposed application, because it is incomplete. Neither the opposed application nor the HGS declaration present any evidence to suggest that VEGF2 as described in the patent application works. The choice of the HGS declarants to discount the scientific evidence and accept the teachings in the patent application about VEGF2 is not based on any scientific principles.

- 2.6 Dr. Gamble's declaration provides another excellent example. There is *direct experimental evidence* underlying Dr. Alitalo's declaration that VEGF2 taught in the application cannot be expressed and secreted. Yet notwithstanding that evidence, Dr. Gamble says, "In my opinion Dr. Alitalo's conclusions represent pure speculation and cannot reasonably be drawn from the results presented in his statutory declaration . . . ." Yet, Dr. Gamble apparently approves of the opposed application and its teachings, even though none of the teachings relating to VEGF2 or its expression or activity are based on reported experimental evidence. I note that when Dr. Gamble critically evaluates the scientific merit of a patent application, her standard is that "whether a patent specification gives examples of biological activity would seem to me to be largely irrelevant." (AJG1 7.18) In my opinion, her standards for scientific evaluation of declaration documents and patent documents are simply irreconcilable. There are other examples as well:

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<sup>2</sup> Dr. Alitalo's first declaration demonstrated that VEGF2 cannot be expressed and secreted as taught in the opposed application. The HGS declarants argued that these results were not reliable because the experiments lacked appropriate controls, for example, but did not present any of their own experiments to show successful expression and secretion. As reported in his second declaration, Dr. Alitalo re-ran his experiments using the controls suggested by HGS and addressing other concerns raised by them, and showed again that VEGF2 cannot be expressed and secreted as taught in the opposed application. In my opinion, his second declaration further validates his first declaration and addresses the criticism raised by HGS. Dr. Alitalo's experiments are really the only evidence on this important subject in this opposition proceeding, because the opposed application contains no evidence of successful expression and secretion of VEGF2 by cells, and HGS provided no such evidence in any of its declarations. (Dr. Power says she expressed and secreted a protein, but the expression experiment that she described is not based on what HGS taught in its application.)

2.6.1 Dr. Gamble "would expect that VEGF2 could be expressed and secreted and would be biologically active (AJG1 at 6.5), even though the application contains no evidence that it can be expressed or secreted. She seems to expect that such expression would be routine in virtually any type of cell, even though the application failed to show expression in any cell types. (AJG1 6.6.) In my opinion, this assertion is simply pure speculation.

2.6.2 Dr. Gamble's opinion is that the mere identification of a DNA sequence and the inherent information it provides, combined with the teachings in the patent specification, "makes possible the manifest therapeutic benefits, which VEGF-2 has to offer, and which will be obtained in the future." (AJG1 at 6.6) She holds these opinions even though the application has no experimental evidence of VEGF2 biological activity, and certainly no evidence that VEGF2 is therapeutic for any disease. In my opinion, in light of the objective scientific evidence, her expressed opinion is pure speculation.

2.6.3 Dr. Gamble finds that the application provides "information concerning gene therapy to provide therapeutic and prophylactic effects against a wide range of different disease states". (AJG1 at 6.8.10) However, the application provides no evidence of gene therapy. Additionally, in all of medicine, there are not currently "a wide range of different disease states" that are treated with gene therapy (using any known gene). Again, in my opinion, in light of the objective scientific evidence, her expressed opinion is pure speculation.

2.6.4 Dr. Gamble finds that the application indicates that VEGF-2 could be isolated from a human cell library from a human embryo, from osteoclastomas, from adult heart, or from adult breast cancer cell lines (AJG1 6.8.3), even though the application fails to demonstrate isolation of VEGF2 protein from any source whatsoever. In my opinion, in light of the objective evidence, her expressed opinion is pure speculation.

2.7 While these are only a few of the examples I could give, I believe my point is clear. Applying a uniform and scientifically objective standard, it would make no sense to

disbelieve a carefully planned and executed experiment by Dr Alitalo, the scientist who is perhaps more familiar with VEGF-C and the VEGF2/VEGF-C gene than any other scientist, if journal publications are any indication, but instead to believe a patent application that speculates widely but has no underlying experimental support.

## B. MIS-QUOTES AND CROPPED QUOTES

- 2.8 The HGS declarants frequently say that they are quoting from or paraphrasing statements from my first declaration, and then responding to them. Occasionally, the quotes are incomplete, or out of context, and fail to capture my full thoughts and opinions on subjects. Sometimes, the paraphrases are simply inaccurate. In some cases, HGS declarants say that are responding to my declaration, when they are not. It is important to read my declaration in context to determine whether the HGS declarants have really responded to what I have said, and not rely on their representations.

## C. FAILURE TO ANALYZE FROM THE STANDPOINT OF SCIENTISTS HAVING COMMON GENERAL KNOWLEDGE IN THE FIELD.

- 2.9 It is my understanding of Australian patent law that most patentability issues are analyzed from the viewpoint of an individual possessed of the common general knowledge in the field of the invention, in Australia, at the time that the patent application was filed. However, this is not always the approach taken by at least some of HGS's declarants.
- 2.10 For example, Dr. Mattick explicitly states that his instructions from HGS were "To review the patent specification and to describe what it would have conveyed to me had I read it in March 1994." (AJM1 at 2.3.1 (emphasis added).) Dr. Mattick was also instructed "To base all comments presented herein on my knowledge as at 8 March 1994, unless I specifically state otherwise." (AJM1 at 2.3.3 (emphasis added).) A vast part of Dr. Mattick's declaration explicitly sets forth this personal point of view, i.e., conclusions that Dr. Mattick personally believes that he would

have drawn or results that he believes he could have personally achieved. (AJM1 at, e.g., 3.33, 4.13, 4.73, and 4.77.) To the extent Dr. Mattick's (or other declarants') prominence and experience and positions gave him knowledge greater than the common general knowledge in Australia in 1994, many of the personal opinions expressed in his declaration may not be relevant to patentability issues.

D. FAILURE TO CONSIDER ALL RELEVANT EVIDENCE

- 2.11 It is not clear from the evidence submitted whether all of HGS's experts considered all of the relevant evidence in reaching their opinions expressed in their declarations.
- 2.12 For example, Ludwig Institute's evidence in opposition included a declaration from Professor Alitalo setting forth evidence that VEGF2 as taught in the HGS application is incomplete and is not expressed and secreted as a growth factor by cells. In my opinion an expert providing an opinion as to the sufficiency and fair basis of the HGS application should have considered evidence about whether the invention works as HGS alleged that it would. It is not clear that Dr. Mattick, Dr. Power, or Dr. Rapoport gave due consideration to the Alitalo declaration.

E. IS VEGF2 THE SAME AS VEGF-C?

- 2.13 Dr. Mattick explains that "HGS' Patent Attorneys have informed me that VEGF-2 and VEGF-C are the same molecule." (AJM1 at 3.3.) Dr. Gamble says "Today, VEGF-2 is identified by the nomenclature VEGF-C. When I read VEGF-2 in the patent specification I understand it to mean VEGF-C" (AJG1 at 3.1.) Dr. Rapoport says, "It is my understanding that VEGF-2 and VEGF-C are terms used to refer to the same molecule." (ATR1 at 13.) Dr. Rapoport uses the two terms interchangeably. (ATR1 at 13.) This position requires clarification.
- 2.14 As I stated in my earlier declaration, it is probably true that VEGF2 and VEGF-C were *derived from* the same human gene. (See OPR1 at 1.5.1.) However, these are

names given by two different research groups to the results of their own research, which differed substantially. The advice from HGS's attorneys or the assumption that these terms can be used interchangeably glosses over substantial differences between the secreted growth factor "VEGF-C" described in patent and scientific literature by Alitalo et al.,<sup>3</sup> and the incomplete "VEGF2" sequence taught in the opposed application, that is apparently never secreted<sup>4</sup> and has never been reported in the scientific literature to have significant biological activity. (I repeat by reference my discussion of the inadequacy of the description of VEGF2 in OPR1 at, e.g., 1.5.1-1.5.2, 4.4-4.7 and 4.11-4.11.2.) To the extent that Dr. Mattick or Dr. Gamble or Dr. Rapoport believed that any of the scientific literature that has been published relating to VEGF-C is relevant to patentability issues for the *incomplete VEGF2* taught in the opposed application, they have based their declarations on incorrect assumptions. The experiments described in Professor Alitalo's two declarations (hereinafter OKA1 and OKA2) demonstrate that the incomplete VEGF2 taught in the opposed application cannot be expressed and secreted.

- 2.15 Dr. Hayward clearly intermingles his knowledge of VEGF-C with the teachings in the opposed application relating to VEGF2. For example, Dr. Hayward declared, "I am aware that VEGF-2 is proteolytically processed upon secretion from cells *in vivo* to form the naturally occurring ligand for the Flt-4 and the KDR/Flk-1 receptors. I would expect a fusion of the 350 amino acid sequence of VEGF-2 to a signal sequence to be proteolytically processed to produce functional VEGF-2." In reality, scientists such as the group led by Alitalo et al. have demonstrated that a heavily processed form of VEGF-C (lacking about 102 amino acids from the beginning (N-terminus) and about 190 amino acids from the end (C-terminus)) binds and stimulates the Flt4 and KDR/Flk-1 receptors. (See, e.g., Documents D70-D74.) The opposed application does not teach that VEGF2 is a ligand for any receptor, and does not teach

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<sup>3</sup> See, e.g., OPR1 at 1.5.3-1.5.4 and Documents D71-D74.

<sup>4</sup> See Alitalo Declaration filed by Ludwig Institute as part of evidence in opposition.



that VEGF2 is processed in a manner similar to VEGF-C, and does not teach to fuse the 350 amino acids of VEGF2 with an extra foreign signal sequence. Instead, the application teaches that full length VEGF2 is 350 amino acids which includes a signal sequence of about 24 amino acids and mature VEGF2 of about 326 amino acids. By contrast, Dr. Alitalo's declarations establish that VEGF2 as taught in the opposed application is not expressed and secreted at all. When HGS determined that VEGF2 in the opposed application was incomplete, and filed a second patent application (Document D43) on the 419 amino acid VEGF2 more than a year later, HGS still failed to teach that VEGF2 was a ligand for Flt4 or any other receptor, and failed to teach the VEGF-C processing that was elucidated by Professor Alitalo's group. Thus, Dr. Hayward's "awareness" of VEGF2 processing relates to Dr. Alitalo's VEGF-C, and not HGS's VEGF2.

- 2.16 Dr. Rapoport devotes a significant part of his declaration to discussing biologically active VEGF-C molecules invented by Alitalo and Joukov. (See, e.g., ATR1 at 13-15.) It is important to remember that Alitalo and Joukov taught VEGF-C "biological activity" (e.g., binding to Flt4 receptor and stimulation of lymphatic endothelia) that were not suggested by (and owe nothing to) the opposed application. The suggestion that the Alitalo work confirms teachings in the opposed application is entirely misleading. (See, e.g., ATR1 at 16.)

#### LACK OF NOVELTY AND INVENTIVE STEP OF CLAIMS OF THE OPPOSED APPLICATION

- 3.0 In Section 2 of the OPR1 declaration, I explained that at least claims 1-4, 13-28, and 34-61 in the opposed application were broad enough to encompass materials and methods that had been taught in the prior art literature that predated HGS's earliest alleged priority date of 08 March 1994. The prior art upon which I drew these conclusions did not teach the exact VEGF2 DNA or deduced amino acid sequences in the figures or sequence listing of the opposed application, but the claims at issue are not limited to exact, full length VEGF2 sequences.

3.1 In this section I explain that the HGS declarants consistently fail to identify any explicit claim language that excludes the prior art that I identified in my first declaration. I address the particulars of the HGS declarations below, but I maintain my original opinion, expressed in OPR1, that most or all of the claims encompass the prior works of others.

3.2 The HGS declarants' principal response to the references cited in my first declaration that demonstrate that the application lacked novelty comprises generalized statements that a practitioner in the appropriate field of research would be able to distinguish VEGF2 fragments, analogs, derivatives, variants, and other genera of molecules claimed in the opposed application from prior art polynucleotides and polypeptides (e.g., VEGF, PDGFA, PDGFB, PIGF, and fragments, analogs, and derivatives thereof disclosed or suggested in the prior art.). A consistent shortcoming of these declarations is the *failure to point to any language in the claims* that defines VEGF2 molecules in a way that excludes prior art molecules.

A. "Fragment, Analogue, or Derivative" Language Fails to Exclude the Prior Art from the Claims.

3.3 Several claims in the opposed application are not limited to the exact VEGF2 sequences because they are directed to a "fragment, analogue or derivative" of a VEGF2 polypeptide (e.g., claim 28) or to a polynucleotide encoding a "fragment, analogue or derivative" of a VEGF2 polypeptide (e.g., claims 1-4, 21). According to the specification, fragments, derivatives, or analogues may be VEGF2 polypeptides modified such that one or more of the amino acid residues of VEGF2 are substituted with conserved or non-conserved amino acid residues, and/or ones in which additional amino acids are fused to the mature polypeptide. (See, e.g., opposed application at pp. 9-10.) Also, polypeptides resulting from deletion of amino acids would still fall within the commonly accepted definition of "fragment, analogue, or derivative." (See also opposed application at page 7, discussing "deletion variants, substitution variants, and addition or insertion variants.")

3.4 In OPR1 I explained that the prior art taught DNA sequences and polypeptides, such as VEGF, PlGF, PDGF-a, and PDGF-b, that differed from the exact VEGF2 DNA and amino acid sequences taught in the opposed application, but still fell within the opposed application's definition of "fragment, analog, or derivative." (See OPR1 at 2.2-2.2.2, 2.3, 2.7.1, 2.7.9, and 2.7.20.) Much of the opponent's evidence in answer is directed to a philosophical discussion of whether these prior art polypeptides, which although they satisfy the literal definition of VEGF2 "fragment, analogue, or derivative", should nonetheless be considered to fall outside the scope of the claims.

3.5 The opposed application says that "it is particularly important that all eight cysteines are conserved within all four members of the family...." These eight cysteines are the only VEGF2 amino acids that the opposed application seems to say should remain unchanged when designing fragments, analogs, or derivatives. However, the application itself admits that the eight cysteines are a feature that VEGF2 already has in common with prior art family members VEGF, PDGFa, and PDGFb. Thus, the opposed application places no restrictions when designing fragments, analogues, and derivatives that would prevent one from arriving at these prior art polypeptides. In other words, all of these prior art molecules (which already contained the eight cysteines) could fairly be classified as VEGF2 fragments, analogues, or derivatives as those terms are used in the opposed application. (See, e.g., OPR1 at 2.2 - 2.2.2.) The claims do not restrict the number of modifications to the VEGF2 sequence that a VEGF2 fragment, analogue, or derivative can contain, so in effect, VEGF2 could be modified until it matched VEGF, PlGF, or PDGF.

3.6 None of the HGS declarants actually contest my analysis that the *claims* fail to recite restrictions on "fragment, analogue, or derivative" that would serve to exclude the prior art polynucleotides and polypeptides such as VEGF, PDGFa, PDGFb, and PlGF. In fact, Dr. Mattick basically admits that *the claims* set forth no maximum number of changes that can be made to a protein that is to be classified as a VEGF2 fragment, analog, or derivative; he simply would not require that as part of the patent laws. (See AJM1 4.37.)

3.7 Instead of finding *claim limitations* that exclude the prior art, the HGS declarants have proposed a subjective “looks more like” standard for *interpreting* the claims that they say would exclude VEGF and the PDGFs from the claims:

3.7.1 For example, Dr. Mattick said: “I would not require the patent specification to set a maximum limit of modifications that can be made to a protein before I could reasonably ascertain whether a protein was a fragment, analogue, and/or derivative of VEGF-2. . . . As soon as a protein starts to look more like VEGF, PDGFa, PDGFb, or PIGF, it would not, in my opinion, be a fragment, analogue and/or derivative of VEGF-2. Thus, I would not regard any of VEGF, PIGF, PDGFa or PDGFb to be VEGF-2 fragments, analogues and/or derivatives.” (AJM1 4.37; see also AJM1 4.26.)

3.7.2 Similarly, Dr. Gamble said: “For something to be a fragment, derivative or analogue of a molecule it must not only share a biological function or activity with VEGF-2 but it must also have homology at the primary amino acid level. Thus, a peptide or polypeptide that more closely resembles VEGF, PDGFa, PDGFb or PIGF, than VEGF-2 at the primary amino acid level would not be a VEGF-2 fragment, derivative or analogue. Such an interpretation is inherent in the meaning of these terms and is consistent with the general manner in which others and I use these terms in everyday scientific language.” (AJG1 at 7.12. See also AJG1 7.25 - 7.27)

3.7.3 Similarly, Dr. Hayward declared that there is not sufficient sequence identity between VEGF2 and PDGF, VEGF, or PIGF for these molecules to be considered derivatives of VEGF2. (See, e.g., ANH1 at 3.8, 4.2.) Dr. Hayward fails to explain what minimum sequence identity is required, and, of course, the claims do not set a minimum sequence identity.

- 3.8 This "looks more like" approach is unworkable. It is highly subjective, vague, has no basis in the opposed application, and would cause the scope of the claims to change over time.
- 3.9 First, the approach that HGS is advocating is contrary to the understanding of patent law given to me by Ludwig Institute's attorneys. It is my understanding that the words of the claim are supposed to define its scope, not subjective "looks like" criteria. The reader is supposed to be able to determine whether or not something falls within a claim by criteria that are reasonably clear.
- 3.10 In fact, the application discloses no clear standards for deciding whether a theoretical analog "looks more like" VEGF or VEGF2, and there are no universal standards in the field of the invention, either. In the context of proteins, the "looks like" criteria could be based on percent amino acid similarity, or on three-dimensional shape, or on size, or more subtle criteria, such as whether amino acids that are believed to be essential for activity are more like those found in VEGF or VEGF2.
- 3.10.1 To give one hypothetical example, the VEGF2 polypeptide has an approximately 190 amino acid stretch that shares homology with a silk protein. (See OPR1 at 4.11.1.3 and Document D71, Joukov et al., EMBO J 16:3898-3911(1997)) Removal of most or all of this large portion of VEGF2 would give the resultant VEGF2 fragment a size much more similar to the size of VEGF. Further, the folded shape of the truncated VEGF2, missing this silk-like domain, might be expected to look much more like the three-dimensional shape of VEGF polypeptides having a similar size. But this fragment has 100% amino acid identity with a portion of VEGF2 and much lower amino acid identity with VEGF. Under Dr. Mattick's "looks like" criteria, its not clear whether or not we have a molecule within HGS's claims, because it is unclear whether size, or shape, or % identity, or other properties are the most important criteria.

- 3.10.2 To give another example, mature VEGF-C has an amino acid sequence nearly identical to a portion of VEGF2, if the approximately 190 BR3P-like amino acids are removed from the C-terminus, and still more amino acids are removed from the N-terminus of VEGF2. This molecule will bind one of the two VEGF receptors (VEGFR-2), whereas VEGF2 will not. (See OPR1 at 4.11.1.3 and Joukov et al., EMBO J 16:3898-3911(1997)) It is unclear under the "looks like" criteria whether one should classify VEGF-C as a VEGF2 analog (because of amino acid sequence identity) or a VEGF analog (because of receptor binding properties). Receptor binding properties would be one of the most important practical criteria for scientists who wished to develop therapeutic applications for a putative growth factor, which clearly seems to be a goal of the opposed application. (It is generally through cellular receptors that growth factors exert their effects in the body.)
- 3.11 The opposed application itself says nothing whatsoever about the "looks more like" standard for delimiting the boundaries of "fragment, analogue, or derivative." In fact, the opposed application fails to mention any standard.
- 3.12 Another troubling aspect of HGS's approach is that the scope of the claims will change with time, as new genes are discovered, because the scope of "fragment, analog, or derivative" would continue to change as more genes are discovered. This problem is best illustrated with two real-world examples.
- 3.12.1 If we placed ourselves back in time to March, 1994, and were given the sequence of VEGF2 and asked to characterize it, the proper answer, according to the "looks more like" standard, would have been to call it a "VEGF fragment, analog, or derivative." The reason for this conclusion *at that time* would have been that the VEGF2 sequence more closely resembled VEGF (30% amino acid identity) than any other known protein at the primary amino acid level. (See Figure 3 of the opposed application.) VEGF2 "looked more like" VEGF than other known molecules. Using the logic of Dr. Mattick and

Dr. Gamble, the VEGF2 sequence would have been a mere analogue or derivative of VEGF.

- 3.12.2 Some time after 1994, the VEGF-D gene and protein were discovered by Achen et al. (See OPR1 at 4.12.1; and Document D67.) VEGF-D has greater amino acid percent identity to VEGF2 than it has to VEGF. Under the HGS "looks more like" criteria, VEGF-D might be considered to fall within the HGS claims (because it is more similar to VEGF2 than VEGF). On the other hand, VEGF-D is a separate human protein in its own right, encoded by its own gene, that owes nothing to the discovery of VEGF2. (See OPR1 at 4.12.1.) It would make no more sense to call VEGF-D an analog or derivative of VEGF2 than it would have made to call VEGF2 a mere analog or derivative of VEGF, when VEGF2 was discovered (due to shared amino acid motifs and allegedly shared biological activities).
- 3.12.3 The logical extension of saying that a newly discovered protein (such as VEGF-D) is *not* a fragment, analog, or derivative of a previously discovered one (such as VEGF2) is that the scope of HGS's patent claims to VEGF2 fragments, analogs, and derivatives becomes narrower each time a new gene family member, such as VEGF-D, is discovered. In other words, before the discovery of VEGF2, all of the analogs that looked more like VEGF than PDGF would have been called VEGF analogs. Then, when VEGF2 was discovered, some of those "VEGF analogs" would have needed to be reclassified as "VEGF2 analogs" because they looked more like VEGF2 than VEGF. In fact, all of the VEGF analogs that looked more like VEGF2 than VEGF or the PDGFs would have been reclassified as VEGF2 analogs. But then, when VEGF-D was discovered, some of those analogs would more properly be classified as VEGF-D analogs, because they were more similar to newly discovered VEGF-D than to VEGF, VEGF2, or the PDGFs.
- 3.12.4 My purpose with this exercise is to show that if you try to apply a "looks more like" standard for interpreting the claims directed to fragments, analogues, and

derivatives, then the breadth of the claim gets narrower every time someone discovers a new member of the VEGF family. The idea that the scope of patent claims changes each time a new VEGF family member is discovered is contrary to my understanding of the clarity requirement of the patent laws. It would cause confusion and uncertainty for scientists trying to interpret the claims.

3.12.5 To summarize, the literal scope of "fragment, analogue, or derivative" claims embraces VEGF, PDGFa, and PDGFb, all of which were known in the field and described in the literature before the priority date of the opposed application. The subjective "looks more like" standard proposed by HGS to avoid this confusion has no support in the application or the claims. It is unworkably vague and causes the scope of the claims to change over time.

**B. VEGF2 "Activity" Language Fails to Exclude the Prior Art from the Claims.**

3.13 A number of the claims of the opposed application use an activity limitation to attempt to define which polynucleotides and polypeptides fall within a claim and which ones do not. I explained in my first declaration that the list of activities ascribed to VEGF2 by the opposed application are merely activities that have been ascribed to VEGF, PDGF, and/or PlGF prior art polypeptides. (See, e.g., OPR1 at 2.3-2.3.5.) For this reason, claim limitations directed to "VEGF2 activity" fail to exclude the aforementioned prior art polypeptides, because the only teachings in the opposed application relating to VEGF2 activity are predictions that VEGF2 will share activities that other scientists had identified in the aforementioned prior art polypeptides. (See OPR1 at 2.3-2.3.5, 2.7.4, 2.7.9, and 4.6-4.8.)

3.14 None of HGS's declarants contest the fact that claims which recite "VEGF2 activity" fail to recite an activity that is unique to VEGF2 and that would serve to exclude prior art polypeptides such as VEGF, PDGFa, PDGFb, and PlGF.



3.15 Dr. Gamble and others ask the Patent Office to accept that VEGF-2 biological activity means "angiogenic activity" and optionally others. (See, e.g., AJG1 7.9-7.11; 7.15-7.18; and 5.14-5.22. See ANH1 at 3.6) There are several problems with their analysis, including the following:

3.15.1 First, even if we accept her selected definition, angiogenic activity fails to exclude prior art vascular endothelial growth factor (VEGF) polypeptides from the claims, because VEGF polypeptides are angiogenic, as admitted in the opposed application (background) and in Dr. Gamble's own papers. (See, e.g., Cockerill, Gamble, and Vadas, Annexure GBC-9 in the HGS evidence in reply, at page 136.) In fact, if angiogenic activity is sufficient for VEGF2 activity, then according to Dr. Gamble's publications, PDGF polypeptides are embraced as well. (See Litwin, Gamble, and Vadas, Annexure GBC-10 of the HGS evidence, at page 105 (PDGF "has roles in wound healing and angiogenesis").)

3.15.2 Second, the claims are not restricted to angiogenic activity, and the application teaches that VEGF2 has numerous activities other than angiogenic activities. (See OPR1 at 2.3.3-2.3.5.)

3.15.3 Third, there is simply no evidence that VEGF2 as taught in the patent application has angiogenic activity (or any of the other activities alleged).

3.16 As a related matter, Dr. Gamble declared that an endothelial growth assay could distinguish VEGF2 polypeptides, fragments, analogues, and variants from PDGF polypeptides, analogues, and variants:

"As an additional point of distinction between VEGF-2 and PDGFa, or PDGFb is that PDGFa and PDGFb do not promote the growth of endothelial cells. Thus had I wanted to distinguish a VEGF-2 fragment, analogue or derivative from any one of these proteins, I could have easily performed one or more assays, such as those identified in paragraph 5.15, above. Such assays could be performed to show that the analogue, fragment or derivative

contained an endothelial cell proliferative activity, while the comparator molecule (such as PDGFa and PDGFb) did not contain such an activity. Such a result would clearly have distinguished a VEGF-2 analogue, fragment or derivative from PDGFa, or PDGFb." (AJG1 at 7.11)

3.17 Dr Gamble's proposition is not correct. First, it is important to re-emphasize that the opposed application teaches that VEGF2 shares biological activities of PDGFa and PDGFb. (See OPR1 at 2.3.3-2.3.5.) Since VEGF2 supposedly has activities other than endothelial growth activities, including PDGF-like activities, it is not clear to me whether the results of only an endothelial cell growth assay would reliably exclude PDGF proteins from the definition of proteins with VEGF2 activity. (See discussion below relating to lack of clarity.) Second, prior art VEGF polypeptides have endothelial cell growth activity, so even if "VEGF2 activity" meant a combination of angiogenic and endothelial cell growth activities, the claims would still encompass prior art VEGF subject matter.

3.18 Dr. Hayward's position regarding "VEGF2 activity" is that he does not understand why a unique defining activity that discriminates VEGF2 from prior art polypeptides (PDGF, VEGF) is necessary at all. He feels that once a substantial portion of the primary amino acid sequence is available, the sequence provides the essential defining characteristic. The answer to Dr. Hayward is that many of the claims (e.g., those that recite fragment, analogue, or derivative and those that recite antibody binding) do not explicitly require the presence of a substantial portion of the primary VEGF2 amino acid sequence. Those claims have neither structural nor functional (activity) limitations that exclude the prior art. If a claim fails to exclude the prior art with structural or functional or other limitations, then the claim encompasses the prior art.

C. "Hybridization" Claim Language Fails to Exclude the Prior Art from the Claims.

3.19 A number of the claims in the opposed application use a hybridization limitation to attempt to define which polynucleotides and polypeptides fall within a claim and which do not. I explained in my first declaration the nature of hybridization experiments, and also that whether two polynucleotides hybridize to each other is dependent on both the relationship of the molecules (complementarity) *and* the conditions under which the hybridization experiment is conducted. (See, e.g., OPR1 at 2.4-2.4.3.) Hybridization experiments are binding experiments with which scientists can determine the relatedness of polynucleotides, and I previously explained that scientists can adjust the experimental conditions (e.g., temperature, ionic strength of solutions used) to increase or decrease the stringency of the experiment and thereby increase or decrease the group of hybridizing polynucleotides. I explained that the HGS claims require no minimum level of stringency that would serve to exclude prior art DNAs/proteins from the claims. (See OPR1 at 2.4-2.4.3, 2.7.5, and 2.7.11-2.7.12.)

3.20 None of HGS's declarants contest the fact that *claims* which recite "hybridization" language fail to *recite hybridization conditions* that would serve to exclude prior art polynucleotides or polypeptides such as VEGF, PDGFa, PDGFb, and PIGF.

3.20.1 Dr. Mattick says that when he reads the hybridization language of the claims *in combination with the description of hybridization set forth in the patent specification*, he would understand "it" to mean that hybridization conditions should be sufficiently specific to exclude known, non-VEGF-2 sequences. He says it would have been routine to perform hybridizations to avoid cross-reactivity with prior art sequences. (AJM1 4.28-4.30, 4.39, and 4.49-52.). Thus, Dr. Mattick does not dispute that the *claims* contain no hybridization stringency limitations whatsoever. Dr. Mattick believes the hybridization claims avoid the prior art because exemplary conditions *in the application* should be interpreted as restrictions on the hybridization claims, even though the claims do not contain the hybridization conditions.

3.20.2 Dr. Hayward discusses hybridization at ANH1 3.9 - 3.12 and 4.11-4.15. He admits that varying the hybridization conditions will affect the number of

inexact polynucleotide matches that will hybridize to a target sequence (ANH1 at 3.10), and, like Dr. Mattick, finds an example of conditions *in the specification* which he says will prevent cross-hybridization (ANH1 at 3.11). He says that cross-hybridization would be unlikely *under suitable conditions such as those provided in Example 1*. (ANH1 at 4.11.) However, he does not dispute that such hybridization conditions are not explicitly recited in the claims themselves.

- 3.21 In view of the comments by the HGS declarants, I have reviewed the application again to determine if the application says that, whenever claims recite hybridization language, the hybridization should be understood to occur under particular conditions. I find no instruction of this nature whatsoever.
- 3.22 My review of other HGS documents further convinces me that the hybridization claims do not exclude the prior art. It appears from other published patent applications that, when HGS intends to limit its claims to certain hybridization conditions, or limit claimed fragments, analogs, or derivatives to those with minimum levels of sequence identity, HGS includes such limitations in the claims.
- 3.23 Filed with this declaration are Exhibits PAWR-1 to PAWR-3, which I understand represent the claims of published patent documents from Human Genome Sciences relating to other DNA and protein inventions. When I examine the claims of those applications, I see explicit limitations like the following:
- 3.23.1 Exhibit PAWR-1 comprises claims from Human Genome Sciences Australian Patent Publication No. 714484, also directed to Vascular Endothelial Growth Factor 2, and having common inventors with the opposed application. I observe the following claiming conventions employed by HGS with respect to that VEGF2 invention, which were not employed with respect to the VEGF2 invention in the opposed application:
- Claim 1, part m: When claiming polynucleotides that hybridize to a reference polynucleotide, HGS specified polynucleotide fragments

which hybridize "under the following conditions: hybridisation in 0.5 M sodium peroxide NaPO<sub>4</sub>, 7% sodium dodecyl sulfate (SDS) at 65°C and washing with 0.5 x SSC, 0.1% SDS at 60°C or equivalent hybridisation stringency." (See also claim 14, 16(m), and 29.)

As I explain in my first declaration, factors such as temperature and solutions used in a hybridization experiment establish (limit) which molecules will "hybridize" and which will not. (See OPR1 at 2.4-2.4.3.) Exhibit PAWR-2 which comprises claims from HGS Australian Patent Publication No. 726486, includes claims reciting similar hybridization conditions. (See, e.g., claims 33-34.)

3.23.2 Exhibit PAWR-3 comprises claims from Human Genome Sciences

Australian Patent Publication No. 708972. I observe the following claiming conventions employed by HGS with respect to that invention, which were not employed with respect to the VEGF2 invention of the opposed application:

When claiming homologues or analogues of a reference sequence, the claims include a "percent identical" limitation which restricts the amount of variation permitted from the original sequence. See, e.g., claim 1, part (h), or claim 6: claim 1 part (n) ("capable of hybridizing . . . and which is at least 70% identical"). Also when claiming variants, claims include a "conservative" limitation which restricts the types of variations that are introduced. See, e.g., claim 15 part (l) ("variant . . . wherein said variant results from conservative substitutions.")

Percent identity language and conservative substitution language serve to limit the amount of variation that is permitted in the molecule.

3.23.3 I have not reviewed these other patents for their merit and express no opinion about the appropriateness of the scope of their claims. Nor do I express an opinion at this time about whether the foregoing types of limitations would cure defects in the claims of the opposed application. I make reference to these documents only to contrast the claim language that HGS used in them to the claim language used in the opposed application. Dr. Mattick would read

the claims of the opposed application to incorporate hybridization or other limitations found in the text of the application. Dr. Mattick's analysis fails to explain why HGS sometimes chooses to include hybridization limitations, percent identity limitations, and other limitations in patent claims and other times does not, if HGS always intends to have its claims limited by such conditions.

D. **Limitations in the Claims Relating to Antibody Binding and Antibodies Fail to Exclude the Prior Art.**

- 3.24 Claims 16-20 and 40-45 of the opposed application recite a genus of any polypeptides that would be bound by any antibody that binds VEGF2. I explained in my first declaration that these claims encompass prior art polypeptides. (See, e.g., OPR1 at 2.5 and 2.74-2.75.) I explained that at least some antibodies that were capable of binding to VEGF2 would be capable of binding to prior art polypeptides, and that consequently, antibody binding limitations of claims failed to exclude the prior art. (See OPR1 at 2.5, 2.7.12, and 2.7.15.)
- 3.25 Dr. Mattick and Dr. Gamble believe that one cannot conclude that these claims are anticipated, in the absence of actual tests showing cross-reactivity, and that the claims cannot be analyzed on a theoretical level. (See, e.g., AJM1 4.31-4.34, 4.40, and 4.48; AJG1 at 7.21.)<sup>5</sup> There are several flaws in Dr. Mattick and Dr. Gamble's analysis.
- 3.26 First, Dr. Mattick contradicts himself only a few pages later, when he declares that "computer programs were readily available in 1994 to generate" information to identify "all of the antigenic sites on the VEGF-2 molecule." (See AJM1 4.83.) It

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<sup>5</sup> In paragraph 4.48 Dr. Mattick speaks of whether the amino acid sequence "preferentially binds" a VEGF2 antibody. I'm not sure what "preferential" binding means, and whatever it means, it is not a requirement of the claims. Only "binding" is required by the claims.

makes no sense to urge that antibody binding defies theoretical analysis if computer programs were readily available seven years ago that could identify all antigenic sites on a protein.

- 3.27 Second, the scientific literature contradicts the conclusions drawn by Drs. Mattick and Gamble. For example, Harlow et al., *Antibodies, a Laboratory Manual*, Cold Spring Harbor Laboratory Press (1988) (cited in the Statement of Grounds and Particulars as a treatise that formed part of the common general knowledge in 1994, and also in OPR1), instructs that synthetic peptides as small as six residues in length "will consistently elicit antibodies that bind to the original protein." (See Harlow at page 76 which is Exhibit PAWR-4 to this declaration). I explained in my original declaration (OPR1 at 2.74-2.75) and Dr. Gamble has confirmed (AJG1 at 7.13) that the VEGF2 sequence and prior art sequences share at least one or two stretches of 6 or 7 amino acid (100%) identity. According to conventional wisdom, these peptides can be expected to elicit antibodies that bind to "the original protein," from which the peptides were derived. In other words, conventional wisdom in the field is that these peptides can be expected to elicit antibodies which would bind both VEGF2 and also bind to the prior art protein (VEGF or PDGF) that shares the common peptide sequence. The personal opinions expressed by Drs. Mattick and Gamble appear to defy conventional wisdom on this subject.
- 3.28 Third, Dr. Mattick's opinion that one cannot meaningfully analyze the claim language relating to polypeptides that would bind an antibody that binds VEGF2 on a theoretical level *compels a conclusion that claims 16-20 and 40-45 of the opposed application are indefinite*. When one examines the HGS application, one sees that the inventors *failed to describe any actual antibodies to VEGF2*, yet the claims seem to encompass the theoretical universe of all antibodies that bind VEGF2. The opposed application does not even set forth the proper antibody binding assay to use to determine whether or not a protein is bound by an antibody that binds VEGF2. Thus, like it or not, the claims at issue *require* a theoretical analysis, if they can be given any meaning at all. If a common sense analysis of antibody science cannot be applied to analyze patent claim language, then patent examiners would be required to perform

absurd amounts of laboratory work or otherwise allow any patent claim relating to antibody binding. The public could never know with any certainty whether it was working with an infringing polypeptide because the public could never test it for binding against all VEGF2 antibodies. (See also discussion in paragraphs 5.4-5.4.4, below.)

3.29 Dr. Mattick's and Dr. Gamble's refusals to draw any conclusions about cross-reactivity and antibodies is another example of the double standard that they have applied to this proceeding. Scientists have decades of experience making and using antibodies, and the experience permits scientists to make predictions about cross-reactivity. For example, scientists would predict that if a first polypeptide has an epitope of about six amino acids, then an antibody to that epitope would probably bind the same six amino acid epitope of a second polypeptide. (See OPR 2.5.) There is a clear incongruity if Dr. Mattick refuses to draw conclusions about antibody binding on a theoretical level, but is willing to believe totally unsupported statements in the opposed application that VEGF2 fragments can be used to treat cancer and all of the other diseases and conditions listed in the opposed application. (See AJM1 4.19-4.24.) Predictions of antibody cross-reactivity due to common epitopes is at least as reliable as predictions of biological activity based on only 22-30% sequence identity, which the HGS application makes and the HGS declarants appear to accept on faith.

3.30 In my analysis of the prior art and novelty issues in my first declaration, I explained that scientists would not normally consider the immunogenicity of a protein to constitute a "biological activity" of the protein. (See OPR1 2.7.4) HGS challenges this position in its evidence in answer. (See, e.g., AJM1 4.45-4.46; ANH1 at 4.13)

3.31 At the outset, I would like to make clear that if immunogenicity constitutes a VEGF2 biological activity, then this only creates an additional reason why claims in the opposed application with "activity" limitations are not novel, as I explain in my first declaration. (OPR1 at 2.7.4.) The VEGF2 polypeptides will be expected to generate some of the same antibodies that are generated by prior art polypeptides.



3.32 HGS has called my explanation (that immunogenicity is not a "biological activity" of a putative growth factor) "spurious." (AJM1 at 4.45-4.46) The plain fact is that when scientists and doctors wish to develop a protein-based therapy, immunogenicity is an undesirable side-effect, and not a "biological activity". By way of illustration, I shall suppose that the biological activity of VEGF2 in the human body is to heal wounds. If one wants to administer VEGF2 as a pharmaceutical to treat wounds, then it is absolutely essential that the VEGF2 NOT be immunogenic. The consequences of VEGF2 being immunogenic (causing the body to make antibodies to VEGF2) could be catastrophic. For example, the antibodies that the body creates would be expected to neutralize the VEGF2 pharmaceutical that was administered. In addition, and more seriously, the body would continue to make VEGF2 antibodies which would neutralize the body's own VEGF2, making it harder or impossible for the body to naturally heal future wounds. In effect, patients being treated with VEGF2 to heal a wound would, as a side-effect, develop a potentially permanent and catastrophic autoimmune disorder. My illustration shows why an immune response to growth factor therapy would be considered highly undesirable. This illustrates by example why persons skilled in the art do not classify immunogenicity of a polypeptide (the ability to raise antibodies to it) together with those functions (e.g., growth factor functions) that are conventionally referred to as biological activities.

3.33 By way of additional illustration, I refer to Exhibit PAWR-5 (Skolnick and Fetrow, "From genes to protein structure to function: novel applications of computational approaches in the genomic era," *TIBTECH*, 18:34 (2000)), a relatively recent review article in the field of genomics that explores such issues as predicting what a protein does based on a protein sequence deduced from a DNA sequence. On page 34, the authors address the question, "What do we mean by protein function, the focus of this article?" The authors suggest function can have many levels and meanings, e.g., depending on whether one is looking at the protein function at a molecular level, physiological or metabolic level, cellular or phenotypic level, or the like. However, the ability to make antibodies to polypeptides is not among them.

E. Reply to Miscellaneous Specific Comments of the Evidence-in Answer

3.34 I offer the following additional comments to particular declarations in the HGS evidence in answer that appear to relate to the issues of novelty and inventive step.

a. AJM1 4.40

3.35 Dr. Mattick declared as follows:

"In paragraph 2.7 Associate Professor Rogers refers to a number of publications, which he says teach the subject matter claimed in the patent specification. I have reviewed each of documents D1, D5, D7, D12, D16, D18, D19, D20, D29, D34, D35, D36, D39 and D41. None of these documents describe VEGF-2." (AJM1 4.40)

3.36 This statement is not relevant to whether the claimed invention is novel. In my first declaration, I explained in detail that *the claims are not limited to VEGF2 molecules*, and that the aforementioned documents describe molecules that fall within the scope of the very broad claims. (See OPR1 at 2.7.)

3.37 Dr. Mattick further declared as follows:

"Further, to the extent that one or more of the claims in the patent specification might include a polypeptide that binds an antibody that binds to VEGF-2 I note that none of these publications describe an antibody that binds to VEGF-2 or establishes cross-reactivity between VEGF-2 antibodies and VEGF antibodies. These documents refer to such subject matter as VEGF, PDGF and PIGF." (AJM1 4.40)

3.38 This statement also is not relevant to the novelty of the polypeptide claims. To the extent that these publications disclose *a polypeptide* that binds an antibody that binds VEGF2, these publications destroy the novelty of the claims which recite this language. The fact that these publications use other names (VEGF, PDGF, PIGF) has no bearing on whether VEGF2 antibodies bind the polypeptides disclosed therein.

b. AJM1 4.43

3.39 Dr. Mattick declared as follows:

"The patent specification clearly identifies an isolated mRNA encoding VEGF-2. For example, Example 1 and Figures 4 and 5 of the patent specification teaches the isolation and detection of an mRNA species (see also the patent specification at page 27, lines 4 to 32). Furthermore, the isolation of VEGF-2 genomic DNA would have been a routine and straightforward task in 1994 for any person of ordinary skill in the field of molecular biology given the information in the patent specification." (AJM1 4.43)

3.40 I establish in my first declaration that the RNA allegedly identified in Example 1 does not appear to be a real mRNA from the gene that encodes VEGF2. (See OPR1 at 4.13.1 and 5.8.10.) Moreover, whatever RNA was observed in Example 1 was observed in a mixture of RNAs that were electrophoresed on a gel. The example does not describe an isolation. With respect to the genomic DNA, I observe that the genomic DNA that encodes VEGF2 encodes a protein of 419 codons, but the patent application only taught about 350 codons. When HGS discovered the missing 69 codons, they filed a second patent application.

c. AJM1 4.53-4.54: AJG1 7.30 - 7.31

3.41 Dr. Mattick declared as follows:

"In paragraph 2.7.18 Associate Professor Rogers states:

"...the claims directed to antagonists of VEGF-2 are not novel over prior art disclosures of forms of the receptors to which VEGF-2 could bind, but could not signal. See Document D27 (disclosing a dominant negative Flk-1 protein)."

"I can find no evidence in document D27 which establishes that the dominant negative Flk-1 protein described could bind VEGF-2. I observe that the dominant negative Flk-1 receptor described in D27 is created by deleting a significant portion of the intracellular kinase binding domain from part of one of the Flk-1 proteins that forms the receptor. Such a deletion may well inhibit binding of VEGF-2. In my opinion, no conclusions can be drawn from D27 about whether a dominant negative Flk-1 protein might serve as a VEGF-2 antagonist." (AJM1 4.53-4.54.)

- 3.42 When Dr. Mattick quoted my first declaration, his cropping changed the context entirely. I prefaced the quoted remark with the statement, "Assuming *arguendo* that VEGF2 as taught in the specification possesses any biological activity that is mediated through cell surface receptors . . . ." VEGF and many other growth factors exert their activity through cell surface receptors. However, there is no evidence in the opposed application that VEGF2 acts as a growth factor for anything, or binds any receptors. That is why I prefaced my remark as I did.
- 3.43 Of course Dr. Mattick can find no evidence in document D27 to establish that VEGF2 binds Flk-1. As I explain in the preceding paragraph, there is no evidence even in the opposed application that VEGF2 binds any receptor or possesses any activity.
- 3.44 However, there is evidence from Dr. Alitalo's group that mature VEGF-C binds and exerts its activities through the receptors Flk-1/VEGFR-2 and Flt4/VEGFR-3. (See OPR1 at 4.11.1.3; Documents 70 and 71.) I raise Document D27 as prior art only if HGS's scientists continue to believe that VEGF2 molecules possess any biological activities mediated through one or both of these receptors.
- 3.45 Dr. Mattick's observation that a significant portion of the tyrosine kinase domain of Flk-1 was deleted to create the dominant negative mutant, and suggestion that the deletion "may well inhibit" binding of VEGF-2 is spurious. The ligand growth factors of Flk-1 are well known to bind the extracellular portion of Flk-1, and not the

intracellular tyrosine kinase domain that was deleted in Document D27 (see, for example, Chiang and Flanagan (Growth Factors 1995;12(1):1-10); Tessler *et al.* (J. Biol. Chem. 1994 Apr 29;269(17):12456-61) and Fuh *et al.* (J. Biol. Chem. 1998 May 1;273(13):11197-204), which are attached hereto as Exhibits PAWR-6, PAWR-7 and PAWR-8, respectively. It has been shown that VEGF-C molecules that can bind Flk-1 can bind a truncated version lacking the Flk-1 tyrosine kinase domain (see, for example, Document D71).

d. AJMI 4.55-4.60; AJG1 7.30 - 7.33

- 3.46 Dr. Mattick and Dr. Gamble declare that claim 50 is free of the prior art on the basis that the claimed antagonists must be "specific for" VEGF-2 polypeptides. (AJMI 4.56; AJG1 7.31) They misread the claim. Claim 50 is directed to an antagonist specific for the polypeptide according to any one of claims 28-48. I establish in my first declaration that claims 28-48 are not limited to VEGF-2 polypeptides, but rather, include prior art polypeptides as well. (See OPR1 at 2.7.9 and 2.7.11-2.7.15.) Because claims 28-48 are not restricted to VEGF2, the antagonist of claim 50 is not specific for VEGF2.
- 3.47 Dr. Mattick and Dr. Gamble misread claims 51-52 in the same way. (AJMI 4.57-4.58; AJG1 7.32-7.33) These claims are not limited to treating a patient with a VEGF-2 protein, but rather with "a polypeptide according to claim 28" or an antagonist thereof. Claim 28 reads on the prior art proteins and antagonists as well as on VEGF-2 (see OPR1 at 2.7.9), and consequently, claims 51-52 read on uses of the prior art proteins or antagonists, as I explain in my first declaration.
- 3.48 In paragraphs 4.59-4.60, Dr Mattick misreads claims 57-61 in the same way, because he believes that the earlier claims to which they refer are limited to VEGF-2 subject matter, which they are not. (See OPR1 at 2.7.21.)

e. AJG1 7.34 - 7.35

- 3.49 In paragraphs 7.34 - 7.35, Dr. Gamble appears to be responding to my explanation (in OPR1 2.7.20) that claim 56 encompasses the prior art. However, she only addresses

one observation regarding the absence of working examples. She does not dispute my analysis or conclusion regarding the claim encompassing the prior art.

**F. Important uncontested aspects of my first declaration.**

3.50 In Sections A-D, above, I explain that the arguments in support of novelty and invention provided by the HGS declarants are not persuasive because they do not relate to what was actually claimed, or because they rely upon unworkable claim interpretations, or other reasons. In Section E I address what I believe to be the only other arguments in support of novelty and invention provided by the HGS declarants.

3.51 To conclude my evaluation of the HGS evidence-in-answer as it relates to novelty and invention, I believe it is worthwhile to identify other uncontested points of my first declaration. In particular, there are many claims that introduce limitations not directly related to VEGF2, such as limitations relating to a heterologous polypeptide (claim 21); limitations relating to vectors, host cells, and methods of production (claims 22-27); limitations relating to use of pharmaceutical carriers (claim 53); limitations relating homo-dimerization (claim 47); and limitations relating glycosylation (claim 48). I explained in my first declaration that none of these claims/limitations confer novelty or invention to any claims that otherwise encompassed the prior art. (See, e.g., OPR1 at 2.7.7, 2.7.8, 2.7.10, 2.7.14, and 2.7.15.) I believe that none of the HGS declarants have contested these opinions expressed in my first declaration.

3.52 Many of the opinions that I offered in my first declaration relating to the issue of inventive step appear to have been uncontested by any HGS declarant. (See, e.g., OPR1 at 3.4 - 3.7.)

**LACK OF FAIR BASIS AND INSUFFICIENCY**

4.1 In Sections 4 and 5 of my first declaration (OPR1), I explained that the opposed application described an incomplete and non-working invention -- a description that was not adequate to practice the invention as claimed, and that certainly was not

commensurate in scope with the breadth of the claims that HGS was asking the Patent Office to grant to them. For example, I explained that there were defects in the application relating to the biological deposit and the description of experiments in the "Examples", and that the results reported in at least one example had been discredited in the scientific literature. I explained that "VEGF2" as taught in the opposed application was incomplete, could not be expressed and secreted as a protein as HGS had instructed in the opposed application, and that there was no evidence that the incomplete VEGF2 had any of the activity alleged in the application. The limited nature of HGS's contribution (sequencing of an (incomplete) cDNA, doing a database sequence comparison, and doing a Northern blot mRNA expression study) was not adequate to predict structure or function of the VEGF2 protein alleged as the invention. It certainly was not commensurate in scope with the amount of experimentation needed simply to make the invention work. I explained that the protein that the human body produces *in real life* from the VEGF2 gene has a structure vastly different from anything taught by HGS in the opposed application, and that the protein's principal function in adult tissues appears to be in regulation of the lymphatic system, a function that completely eluded the HGS inventors and was not suggested in the patent application. (The protein's structure and function were elucidated by Alitalo et al., a group that has since been awarded multiple patents on their work.) I explained that many of the HGS claims were not based on the application as it was filed, but rather, were an attempt to capture inventions such as those of Alitalo et al. that were published during the pendency of the opposed application before the Patent Office, but that owed nothing to the contributions embodied in the opposed application.

- 4.2 In this section of my declaration, I reply to the HGS evidence-in-answer that appears to relate to the issues of Lack of Fair Basis or Insufficiency. The declarations offered by HGS usually failed to identify which issue they were answering. Accordingly, I have attempted to reply to all such issues in a single section of this declaration.

A. ADMISSIONS AND UNCONTESTED EVIDENCE

- A1. Admissions that the invention was incomplete.

- 4.3 Many of the HGS declarants have admitted for the record that VEGF2 as taught in the patent application is incomplete. Thus the opposed application did not provide the public with a complete invention. The HGS declarants admit that the beginning of the VEGF2 polynucleotide and polypeptide molecules, including the true VEGF2 signal sequence, was missing from the application as filed, and thus, that the teaching in the application that the first 24 of 350 amino acids represent a signal sequence, with mature VEGF2 being 326 amino acids, was simply incorrect.

4.3.1 Dr. Mattick: "the fact that the signal sequence information was incomplete" (AJM1 at 4.13.)<sup>6</sup>

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<sup>6</sup>Dr. Mattick admitted in his declaration that "An important difficulty that researchers faced in 1994 (and still face today) is the process of determining what a new gene encodes. This involves careful consideration and scientific training, and it is not a simple or straightforward process. Importantly, the isolation of a DNA sequence does not guarantee sufficient information to establish whether the sequence encodes a protein or if it does, the nature and function of the protein it might encode. Such information had to be determined in 1994 (as it is today) by a researcher using scientific skill, their experience, their knowledge and often a wide range of different analytical and experimental tools." (AJM1 at 3.13) Dr. Mattick declared that "once a DNA sequence had been cloned, further manipulations of that sequence would be relatively routine practice. Moreover once a protein sequence had been identified there were many routine methods available for analysing that protein." (AJM1 at 3.14.) When reading the declarations filed by HGS, it is important to remember that the inventors HAD NOT YET CLONED THE COMPLETE VEGF2 GENE. (In fact, the applicants filed a second patent application, more than a year later, when the inventors realized that the VEGF2 gene was incomplete.) Many of the techniques available for analyzing DNA and protein would have been fruitless if applied to VEGF2 taught in the opposed application, because VEGF2 was incomplete.



4.3.2 Dr. Gamble: "I note that a portion of the full length VEGF-2 sequence, which is not disclosed in the patent specification, is part of the NH2 (amino) terminal end of the full length polypeptide sequence. This equates to 69 amino acids . . . . [The VEGF-2 signal] sequence is located among the 69 amino acids that were not disclosed in the patent specification." (AJG1 at 6.4.)

4.3.3 Dr. Hayward: "The patent specification discloses 350 amino acids of the VEGF-2 sequence whereas it has subsequently been determined that VEGF-2 has 419 amino acids. The missing amino acid sequence is now known to contain the signal sequence that directs secretion of VEGF-2 from the cell." (ANH1 at 3.13.)

4.3.4 Dr. Rapoport: "[T]he 350 amino acid form of VEGF2 corresponds to amino acid residues 70 to 419 of the 419 form of VEGF2." (ATR1 at 8).

4.4 Many of the HGS declarants then devote substantial material to explaining that they would have eventually experimented sufficiently to make alternate molecules (e.g., foreign signal sequences attached to either a 350 amino acid VEGF2 or a 373 amino acid VEGF2) that the opposed application simply fails to teach. Such experimentation is beyond the teachings of the application, which is limited to relatively straight-forward partial cloning and sequencing work..

4.5 In fact, a strikingly large percentage of the evidence-in-answer is devoted to discussions of experiments that the declarants might have thought to have performed back in 1994, if provided with the teachings of the opposed patent application, in order to try to make or use the VEGF2 invention. I have tried to catalogue some of the numerous paragraphs in the evidence-in-answer devoted to such "obvious experimentation" to demonstrate its magnitude. (See Exhibit PAWR-9 to this declaration.) The extensive experimentation that HGS's experts would have needed

to perform validates my previous declaration setting forth that the opposed application lacks a disclosure adequate to place the public in possession of what is claimed as the invention. The large quantity of experimentation suggested, which the declarants for the most part have not actually performed to see if it would work, would not be needed at all if the invention worked in the manner described in the opposed application. I discuss some of the details of the proposed experiments further below.

- 4.6 Especially noteworthy is the large amount of experimentation devoted to figuring out that the application was wrong, and how to correct it. I explain in detail below that, even if all of the experimentation would have eventually resulted in a working invention, it would no longer be the invention of the opposed application. The experimentation suggested by HGS's declarations proceeds in a different direction.

A.2 Admission that VEGF2 is too distantly related to prior art molecules to reliably predict structure of function.

- 4.7 The opposed application is devoid of any evidence of VEGF2 activity. Instead, it predicts that VEGF2 will have the activities that others had shown for VEGF or for the PDGF's. In my first declaration I carefully explained that 22-30% amino acid identity between proteins would not have been considered sufficient to predict biological activity, and gave many examples. (See OPR1 at 4.6.3 - 4.6.5.) None of HGS's declarants explicitly disagreed with this position. In a statement that could be construed as partial agreement, Dr. Hayward declared "that there is not sufficient sequence identity between VEGF-2 and PDGF, VEGF and PIGF (as is clearly demonstrated in Figure 2) for these molecules to be considered derivatives of VEGF-2." (ANH1 at 3.8; see also ANH1 at 4.11 ("lack of detectable homology").

- 4.8 In fact, Dr. Gamble characterizes the homologies at the amino acid level between VEGF, PDGF $\alpha$ , PDGF $\beta$ , and VEGF-2 as very low, with significant differences between the molecules, and very few stretches of significant contiguous amino acid sequences:

When regard is had to the information provided in the patent specification one observes that the homologies at the amino acid level between VEGF, PDGFa, PDGFb and VEGF-2 are very low. The identity between VEGF-2 and each of VEGF, PDGFa, PDGFb is 30%, 23% and 22% respectively (see page 5 of the patent specification). Further, there are very few contiguous sequences of amino acids that are identical between VEGF-2 and each of VEGF, PDGFa or PDGFb (see Figure 2 of the patent specification). In fact, the largest single contiguous sequence of amino acids is only seven amino acids in length and is found in the signature motif, which is identified by a box in Figure 2. After that stretch of residues there is one other stretch of six contiguous amino acids but no other significant contiguous amino acid sequences that share identity between VEGF-2 and VEGF, PDGFa or PDGFb. Thus, at the primary amino acid level there are significant differences between VEGF-2 and VEGF, PDGFa or PDGFb. (AJGI 7.13.)

- 4.9 I believe her characterization supports nicely the point that I made in my first declaration, that the low similarity and significant differences between VEGF2 and VEGF or the PDGF's would not permit reliable prediction of structure or function.
- 4.10 Similarly, Dr. Hayward says that "there is not sufficient sequence identity between VEGF-2 and PDGF, VEGF and PIGF (as is clearly demonstrated in Figure 2) for these molecules to be considered derivatives of VEGF-2." I interpret his statement to be an agreement that 22-30% sequence identity is too low for a person to have concluded that proteins would share the same function.

- 4.11 In my first declaration I explained that the examples in the application contained errors and omissions. (See, e.g., OPR1 at 4.13-4.13.5.) My analysis is essentially uncontested by the HGS declarants. In some instances, HGS's declarants confirm that there are errors and omissions. (See, e.g., ANH1 4.27; AJM1 4.90-4.92.)

**B. MERE CATALOGUING IS NOT EVIDENCE AND DOES NOT ANSWER MY INITIAL CRITICISMS OF THE OPPOSED APPLICATIONS**

- 4.12 Significant portions of the evidence-in-answer are devoted to highlighting the many general molecular biological techniques allegedly taught in the opposed application. (See, e.g., AJM1 at 3.12-3.19; 3.29, 3.31, 4.71; and 4.83; AJG 6.8-6.8.11; ANH1 at 3.6, 3.11, 3.21.) I acknowledged in my first declaration that the HGS application contains such "stock" teachings that HGS apparently chooses to include in many of its molecular biology patent applications, irrespective of the inventors and irrespective of the invention described and claimed. (OPR1 at 4.6.2.1.) I also set forth in detail many specific deficiencies of the opposed application which are not cured by regurgitation of standard molecular biological techniques. I maintain my opinion that the invitation in the opposed application to conduct further experimentation using standard molecular biological techniques does not cure the failure to adequately describe the VEGF2 invention in a manner commensurate with which HGS has attempted to claim it. No matter how much attention HGS draws to its stock teachings, they remain nothing more than an *invitation to experiment* and not a teaching of a complete VEGF2 invention. The amount of experimentation that HGS has left for the Australian public to perform in order to determine the true structure of the VEGF2 gene and protein, a working method of expressing and secreting the protein, and a proper identification of its biological activities vastly outweighs the amount of experimentation that HGS conducted before it filed its application on the incomplete VEGF2 gene. The experimentation is not merely routine work to make the invention work in the manner taught by the application. Rather, it is experimentation to discover something that is not described in the application, and how to make it work.

4.13 Significant portions of the evidence-in-answer are devoted to repetition of various predictions in the patent application that are unsupported in the application by scientific evidence, or even by a sound basis for prediction. I have attempted to catalogue some of these portions of the evidence-in-answer in Exhibit PAWR-10 to this declaration. I acknowledged in my first declaration that the HGS application contains such predictions. (OPR1 at 2.3.3 and 4.6.5.) I also set forth in detail that these were nothing more than predictions, lacking any apparent basis in scientific experiments. (OPR1 at 4.6.3.1 and 4.6.5.) I also set forth in detail that there was no accepted scientific basis for the predictions. (OPR1 at 4.6.3.1 and 4.6.5.) The evidence-in-opposition also included scientific evidence that many of the predictions were simply wrong. (OPR1 at 4.6.4 and 4.6.5.) The many specific deficiencies of the opposed application are not cured by regurgitation of the inventors' predictions in the evidence-in-answer, without critical evaluation of whether they have basis in fact. I maintain my opinion that the invitation in the opposed application to conduct further experimentation to determine how to make and use the invention, by seeing which (if any) predictions were correct does not cure the failure to adequately describe the VEGF2 invention in a manner commensurate with which HGS has attempted to claim it.

**C. THE LARGE BODY OF EXPERIMENTATION EMBODIED IN THE HGS EVIDENCE CONCLUSIVELY PROVES THAT THE OPPOSED APPLICATION WAS INCOMPLETE AND INADEQUATE.**

4.14 In this section I explain in greater detail that much of HGS's evidence-in-answer actually supports Ludwig Institute's opposition by confirming that the invention does not work as taught, and demonstrating that substantial experimentation and ingenuity would have been required to make the invention work. In many cases, the proposed experiments would have required ingenuity to contravene plain teachings in the application, after experimentally determining that the invention does not work as taught. In other cases, the ingenuity was of sufficient character that at least HGS believed that it warranted the filing of additional patent applications. In other cases,

the ingenuity was of sufficient character that other parties filed and were awarded patents on the work.

C1. Experimentation Needed to Make VEGF2 Polypeptides

- 4.15 I have reviewed the two declarations of Professor Alitalo where he performed controlled experiments to determine whether the approximately 350 amino acid VEGF2 taught in the opposed application can be expressed and secreted as a mature VEGF2 in the manner taught by the application.
- 4.16 In my opinion, Professor Alitalo's experiments were designed as an accurate reflection of what the patent application teaches to scientists in the field of the invention. The application teaches that the approximately 350 amino acid VEGF2 comprises a leader (signal) sequence of approximately 24 amino acids that would direct secretion of a mature VEGF2 of about 326 amino acids. Dr. Alitalo's experiments were designed to see if the encoding cDNA could be expressed and secreted as taught in the opposed application.
- 4.17 Dr. Alitalo's experimental results demonstrate that VEGF2 is not expressed and secreted as taught in the application. The design of the experiments was sound, and the results are clear. Knowing what we now know in year 2001 about VEGF2, an obvious explanation for the lack of expression is that the construct in the patent application lacks a signal peptide. The opposed application teaches an incorrect and nonfunctional signal peptide. It is not clear to me whether this error was ever brought to the Patent Office's attention before the opposition proceeding instituted by Ludwig Institute.
- 4.18 In fact, I think the declarations filed by HGS reflect silent recognition that the application was completely wrong about VEGF2, because the HGS declarants admit that the true signal peptide is missing, and then discuss alternative ways of expressing VEGF2 that are NOT taught in the application.

4.19 For example, Dr. Mattick admits "the fact that the signal sequence information was incomplete" at paragraph 4.13. In fact, he begins his detailed explanation of how he might have successfully expressed VEGF2 *with the assumption that an attempt to express the VEGF2 protein using the putative secretion signal sequence identified in the patent specification would not work.* (See AJM1 4.5, and ensuing discussion in paragraphs 4.6-4.13; see also AJM1 4.76-4.78.) In the ensuing discussion, Dr. Mattick goes on to explain how he would have deduced from the opposed application to express an approximately 373 amino acid VEGF2 sequence using a heterologous signal peptide (AJM1 4.7-4.8), even though he acknowledges in the very next paragraph that the application teaches only to attach a heterologous signal to "the mature sequence." (AJM1 4.9)

4.20 The 373 amino acid construct that Dr. Mattick says that he would eventually have made is not described in the application at all. The application actually only teaches an approximately 350 amino acid full length VEGF2 and mature VEGF2 of 326 amino acids. Viewed in the manner most charitable to HGS, Dr. Mattick has said that, after determining (or being told) that the invention described in the patent application does not work, he might have been able to design some experiments to make an embodiment that is not described anywhere in the application, but that he thinks could work. And there is no indication that he has performed these experiments to see if they really do work.

4.21 Dr. Gamble makes the same admission as Dr. Mattick that the VEGF2 invention is incomplete: "I note that a portion of the full length VEGF-2 sequence, which is not disclosed in the patent specification, is part of the NH2 (amino) terminal end of the full length polypeptide sequence. This equates to 69 amino acids . . . . [The VEGF-2 signal] sequence is located among the 69 amino acids that were not disclosed in the patent specification." (AJG1 at 6.4.) Like Dr. Mattick, Dr. Gamble assumes (with hindsight) that she would have had knowledge of this defect in the patent application, and explains that "The mere fact that the sequence disclosed in the patent application does not include the first 69 amino acids of the full-length VEGF-2 sequence would not dissuade me from attempting to express the sequence disclosed in the patent

specification with a heterologous signal sequence.” (AJG1 at 6.5.) Thus, Dr. Gamble apparently would have tried to attach a heterologous signal sequence to the approximately 350 amino acids of VEGF2 disclosed in the patent application. Dr. Hayward says he would have used a similar approach. (See ANH1 at 4.20.)

4.22 Dr. Hayward’s declaration of how he would have made VEGF2 polypeptides is redundant to the Gamble and Mattick declarations. Dr. Hayward, like Dr. Mattick and Dr. Gamble, admits that the VEGF2 taught in the patent application is incomplete, missing a large portion of the amino terminus which “is now known to contain the signal sequence . . . .” (ANH1 3.13.) Like Dr. Mattick, he says that he would have read the application as providing 373 amino acids, even though it explicitly states that VEGF2 is only 350. (ANH1 at 3.14.) Like Dr. Gamble, he says that he would have experimented and eventually attempted to express the 350 amino acids with a heterologous signal sequence, even though the application taught that the 350 amino acids already contained a signal sequence. (ANH1 at 3.20-3.26) Like Dr. Mattick, he says that *if* the 350 amino acid VEGF2 as taught in the patent application could not be expressed and secreted, *then* he would have begun experimenting to attempt to figure out what was wrong. (ANH1 at 3.17)

4.23 Dr. Rapoport provides a similar, redundant explanation. He says that all secreted proteins have easily recognizable signal sequences (ATR1 at 4-6), but does not see such a sequence in VEGF2 of the application. (ATR1 at 10.) Instead of concluding that VEGF2 is not secreted, he says he would have had great confidence that VEGF2 was secreted, and would therefore have attached a “strong” foreign signal sequence to 350 amino acid VEGF2. (ATR1 at 12.) He declares that “this approach is specifically taught in the HGS patent specification” at page 14, but it is not. Nothing in the opposed application would fairly be read to teach attaching a heterologous signal sequence to the portion of VEGF2 that was identified in the application as a signal sequence. Such signal peptide redundancy makes no sense when recombinantly expressing polypeptides.



- 4.24 The experimentation suggested by all of these declarants is not taught or even suggested by the opposed patent application, but rather is driven by hindsight knowledge of information published after the opposed application was filed. (See discussion below (subpart D) at paragraphs 4.55-4.66, repeated here by reference.)
- 4.25 Before leaving the topic of the VEGF2 expression experiments suggested by Drs. Mattick, Gamble, Hayward and Rappaport, I believe it is important to re-emphasize that they do not even attempt to reproduce the invention described in the opposed application. In fact, it is ironic that the four experts would read an application that taught mature VEGF2 is 326 amino acids, and then be motivated to make a construct comprising a foreign signal peptide attached to VEGF2 of 350 or 373 amino acids. The results of such experiments should have no bearing on the adequacy of the opposed application's teachings, because they are neither disclosed nor suggested in the opposed application. As I explain above, the approaches suggested by all of the HGS declarants reflect an unspoken admission that the invention does not work as taught in the opposed application. Whether or not the HGS declarants approve of Professor Alitalo's experiments described in his two declarations, they seem to recognize that his results are accurate, because they have abandoned the teachings of the application and are now advocating expression strategies that are not in the application.
- 4.26 Dr. Mattick, Dr. Gamble, Dr. Hayward and Dr. Rapoport all cite U.S. Patent No. 6,130,071, issued to Alitalo et al., as evidence that VEGF2 could be produced using the experimental approaches he has outlined in his declaration. (See AJM1 4.12; AJG1 6.5; ANH1 4.20; and ATR1 at 13)<sup>7</sup> In my opinion, the Alitalo patent only serves as evidence that all of the extra work that the HGS declarants are describing is *inventive work by another*, and not routine work that should be credited to the opposed application of HGS. I observe in the "References Cited" portion of the

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<sup>7</sup>Dr. Rapoport also relies on Dr. Alitalo's published journal articles, relating to VEGF-C. (See, ATR1 at 13-15).

relevant patent the citation of Documents D43, U.S. Patent Nos. 5,932,540, 5,935,820 (Documents D92 and Exhibit PAWR-11 respectively), and International Publication No. WO 95/24473 (Exhibit PAWR-12), all of which are related in some manner to the opposed application. In fact, WO 95/24473 appears to be identical to the opposed application. Ludwig Institute's attorneys have explained to me that, because these documents are listed in the "references cited" section, the documents were considered by the U.S. examiner when deciding to issue U.S. Patent No. 6,130,071. The fact that a U.S. patent examiner considered the invention of 6,130,071, filed in 1997, to be patentable after considering the contents of the opposed application (in the aforementioned documents), suggests to me that the work described in that patent is inventive work of others, and owes little or nothing to the teachings of the opposed application. Patented work of others can not reflect the efforts of routine experimentation.

4.27 Moreover, it is not clear to me how the content of U.S. Patent No. 6,130,071 could be argued to support the adequacy of the disclosure of the opposed application in any way. The biologically active VEGF-C forms described in the 6,130,071 patent are NOT the 350 or 326 amino acid forms of VEGF2 taught in the opposed application. (Dr. Gamble represented to the Patent Office that they were in AJG1 6.5) Nor are they the 373 amino acid form that Dr. Mattick and Dr. Hayward believe they would have thought to create after reading the opposed application. Nor are activities such as Flt4 receptor stimulation or lymphatic endothelial cell growth factor of the Alitalo patent attributable to the opposed application. Dr. Rapoport premised his analysis on the "understanding that VEGF-2 and VEGF-C are terms used to refer to the same molecule." (ATR1 at 13). At least in the context of the present application, which taught an incomplete VEGF-2, and mistaught the mature molecule, this premise is clearly incorrect. (See paragraphs 2.13-2.16, above).

4.28 Also, it should be emphasized that Dr. Alitalo's research group had identified the full length 419 amino acid prepro-VEGF-C and had characterized its biological activities at the time that they filed their 1997 patent application and published their 1997

paper. The notion that this work should be credited to the opposed application, which taught an incomplete protein that is not expressed and secreted and that has never been reported in the literature to have any activity, is unacceptable.

4.29 Dr. Mattick declares that the VEGF2 sequence provided in the patent application "would have allowed me and I believe any person of ordinary skill in the field of molecular biology in 1994 to design specific strategies to obtain any polynucleotide sequence (ie gDNA, mRNA or cDNA) encoding VEGF-2." (AJM1 at 3.34) Dr. Hayward makes a similar declaration. (ANH1 at 3.26 and 4.18.) Of course, "designing strategies" is merely designing experiments for further research, and no guarantee of results. It is only necessary because the invention does not work the way that HGS taught in the opposed application. When HGS eventually isolated a full-length VEGF2 sequence of 419 amino acids, they thought the new result was significant enough to file a brand new series of patent applications. [See Documents D44-46 and discussion at OPR1 paragraphs 1.5.1.1-1.5.3.] If Dr. Mattick and Dr. Hayward think that "any" VEGF2 polynucleotide sequence was within routine reach by a person of ordinary skill, they are disagreeing with the inventiveness in HGS's second series of VEGF2 patent applications.

4.30 Dr. Mattick and Dr. Hayward also declare that the patent application's teachings regarding VEGF2 were sufficient to identify and isolate VEGF2 from other species. (See AJM1 4.93-4.94; ANH1 at 4.19.) The fact is, the teachings regarding VEGF2 are not even sufficient to provide the public with complete human VEGF2. (See OPR1 at, e.g., 1.5.1.1 and 4.11-4.11.1.3.) When HGS eventually discovered full length human VEGF2, they believed that the discovery was of sufficient magnitude to warrant another series of patent applications. (See Documents D44-D46.) It would have been a much greater leap from incomplete human VEGF2 to complete VEGF2 from other animals. Thus, I cannot agree with Dr. Mattick and Dr. Hayward's evaluation.

## C2. Experimentation Regarding VEGF2 Biological Activity

- 4.31 The preceding paragraphs relate to all of the experimentation that HGS *admits* through its evidence in answer was necessary simply to express VEGF2 polypeptides.<sup>8</sup> That is only *part* of the experimentation that would have been needed to practice the VEGF2 invention, because polypeptides *per se* are not necessarily useful for anything more than a curiosity for further research. It is equally clear from the evidence in answer that further experimentation was needed to figure out exactly what VEGF2 biological activity is. I originally discussed inadequacies of the teachings of the opposed application with respect to VEGF2 biological activity in OPR1 at 2.3.2, 2.7.4, 3.4.2, 4.6, 4.6.1-4.6.5, 4.8, 5.4, 5.5, and 5.9.
- 4.32 Dr. Mattick discusses VEGF2 biological activity at, e.g., AJM1 4.14-4.24. Dr. Gamble discusses VEGF2 biological activity at, e.g., AJG1 5.14-5.22, 6.7, 7.8-7.11, 7.15-7.18; and 7.28-7.29. Dr. Hayward discusses biological activity at, e.g., ANH1 at 3.6, 3.27-3.36, and 4.3-4.10.
- 4.33 In paragraph 4.15, Dr. Mattick selects ONE PASSAGE of the opposed application relating to *in vitro* expansion of vascular endothelial cells and says that he would have understood from it that an expected activity of VEGF-2 is to promote growth of vascular endothelial cells in culture. In paragraph 4.16 Dr. Mattick says that, "had I wanted to examine a secreted VEGF-2 protein using other assays relevant for activities specified in the patent specification (see below) I would have identified someone working in the vascular biology or endothelial biology field and I would have asked them for advice about the types of angiogenic assays that were available in 1994 and how to set up the assays, or I would have asked whether they would be prepared to collaborate with me in my research by testing the protein that I had produced." Dr. Gamble similarly selects testing for endothelial cell growth and/or angiogenic activity. (AJG1 5.14-5.22, 6.7, 7.8-7.11, 7.15-7.18, and 7.28-7.29.) Dr. Hayward selects these two functions and a few others. (See, e.g., ANH1 at 3.28-

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<sup>8</sup>And, as I explain, the polypeptides that HGS's experts would make are not the one taught in the opposed application.

3.29.) Dr. Mattick says he could have used an endothelial growth activity to verify the production of VEGF2. Dr. Gamble says she could have tested VEGF2 activity with such an assay. Dr. Hayward says the testing would have been routine. I find several flaws in their analysis.

4.34 HINDSIGHT. As I explain in detail in my first declaration, endothelial cell growth is only one of a huge number of alleged uses for VEGF2 in the opposed application. Applying the reasoning of the HGS declarants, each one of the uses could have given rise to a number of activity assays. The HGS declarants apparently selected endothelial cell culturing because they (e.g., Dr. Mattick and Dr. Gamble) believe that HGS's subsequent work (e.g., embodied in the HGSII application, Documents D44-D46) shows that this is a valid activity.<sup>9</sup> Even if endothelial cell growth were a true activity of VEGF2, there is nothing *in the opposed application* proving that endothelial cell culture is a good selection, as opposed to, e.g., a bone growth assay, a ligament growth assay, a cementum growth assay, or a collagen growth assay, just to name a few. (See opposed application at p. 17, first paragraph.)

4.35 Dr. Mattick alleges that VEGF2 activity for promoting growth of endothelial cells in culture has been validated:

"I note that such an activity has subsequently been shown to be something possessed by VEGF-2. I refer to HGS Patent Application 60467/96 (714,484), which shows that VEGF-2 exhibits proliferative effects on vascular endothelial cells (see page 42, line 32 to page 43 line 25 and Figures 8 and 9)." (AJM1 4.15)

4.36 Dr. Mattick should have, but failed to, qualify this remark by explaining that the VEGF2 purportedly tested in HGS's *second* VEGF2 patent application is a *different* VEGF2 than the VEGF2 that HGS taught in the opposed application. While Dr.

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<sup>9</sup>This subsequent work related to polypeptides that were not taught in the opposed application and are not informative about the activity of VEGF2 polypeptides at issue in this proceeding. See paragraph 4.36.

Mattick may not think that this difference is significant, it appears to me that HGS felt that it was significant enough to warrant filing a second VEGF2 patent application. Drawing a conclusion about 326 amino acid mature VEGF2 taught in the opposed application based on data derived from what was allegedly an approximately 374 amino acid VEGF2 is precisely the type of scientific reasoning that HGS's declarants (e.g., Dr. Gamble) would rebuke as highly speculative. The excerpts cited by Dr. Mattick do not permit the conclusion that VEGF2 *taught in the opposed application* is useful for endothelial cell culture.

4.37 Dr. Mattick and Dr. Gamble cite no evidence whatsoever concerning the validation of an angiogenesis assay for the VEGF2 taught in the opposed application.

4.38 The only alleged scientific evidence of VEGF2 biological activity cited by Dr. Mattick, Dr. Gamble, or Dr. Hayward is in Example 1 of the application:

4.38.1 Dr. Mattick: "Further the patent specification provides in Example 1 Northern Blot data (see figure 4) showing that VEGF-2 is over-expressed in breast cancer cell lines. This result indicates to me that VEGF-2 is biologically active in tumours." (AJM1 at 4.18.)

4.38.2 Dr. Gamble: "... confirmation that the VEGF-2 sequence described in the specification is expressed in vivo, and therefore likely to indicate a biological activity, is provided in the Examples of the specification where it is shown by Northern blotting that a number of malignant breast tumour cell lines over express VEGF-2. The over expression of VEGF-2 in breast cancer cell lines while absent in a normal breast sample suggests a role of VEGF-2 in tumor development perhaps by promoting the growth of new blood vessels, as was observed for VEGF. (AJG1 7.17. See also AJG1 7.28 - 7.29)

4.38.3 Dr. Hayward: "In Example 1 in the patent specification the inventors demonstrate that VEGF-2 is over expressed in vivo in a number of malignant breast tumour-cell lines. This result is, I believe, indicative of VEGF-2 biological activity. It suggests to me that VEGF-2 plays a role in tumour development possibly by promoting new blood vessel growth similar to VEGF. Additionally, it is apparent to me from reading the patent specification as I believe it would be to others in my field that VEGF-2 is a growth factor that is related to VEGF and is likely to play a role in the regulation of endothelial cell mitogenesis." (ANH1 at 4.6)

4.38.4 There are several problems with Dr. Mattick, Dr. Gamble, and Dr. Hayward's conclusions.

4.39 FAULTY EXPERIMENT. I explain in detail in my first declaration that the weight of published scientific experiments indicate that the gene that encodes VEGF2 produces an RNA message substantially bigger than 1.6 Kd, the size reported in Example 1. (See OPR at 4.13.1..) It is not clear to me that Example 1 even contains valid data.

4.40 WHAT IS THE VEGF2? Even if VEGF2 is active in tumors, Example 1 fails to address the question of what VEGF2 molecule we are talking about. The VEGF2 taught in the opposed application is incomplete, and I have never seen any published evidence that the incomplete VEGF2 is expressed and/or secreted in any human cells (healthy or cancerous). Dr. Alitalo's two declarations provide experimental evidence that VEGF2 as taught in the opposed application is not expressed and secreted by cells. Dr. Alitalo's published body of work relating to VEGF-C indicates that molecules very different from VEGF2 (as taught in the opposed application) are expressed *in vivo*. (See, e.g., Documents D71-D74.) Just because something might be happening with VEGF2 in tumors does not mean that the incomplete VEGF2 taught in the patent is involved. To the extent that a VEGF2-like molecule is

involved in breast cancer, the molecule might be the 419 amino acid VEGF2 that was the subject of HGS's second VEGF2 patent application, or the VEGF-C proteins that were the subject of Professor Alitalo's patent filings. A Northern hybridization study, even if performed correctly, would not indicate to a scientist what protein forms are present.

4.41 WHAT IS THE ACTIVITY? I shall assume in this paragraph that Dr. Mattick, Dr. Gamble, and Dr. Hayward are correct in concluding from Example 1 that VEGF2 is biologically active in tumors. Unfortunately, a scientist cannot say from a simple Northern hybridization experiment *what that activity actually is*. A correctly run Northern hybridization study tells a scientist whether a particular RNA is present in a sample, and possibly how much of that RNA is present in the sample. Without further experimentation, one cannot know whether the tumors are secreting VEGF2 as a growth factor for tumor cells (autocrine growth factor), or secreting VEGF2 to inhibit further growth of tumor cells, or secreting VEGF2 to stimulate or inhibit growth of some other tissue type, or secreting VEGF2 to kill nearby healthy cells to make room for tumor growth, or secreting VEGF2 to recruit some other cell type to the tumor, or secreting VEGF2 to inhibit immune cells such as tumor infiltrating lymphocytes from attacking the tumor, or any of a myriad of other activities. The scientific reality is that a Northern blot study provides only indirect evidence that a cell might be making a particular protein. It provides no evidence whatsoever as to what activity, if any, the protein is having. To paraphrase Dr. Gamble, any conclusion about VEGF2 activity from a Northern hybridization study, represents "pure speculation" and "cannot reasonably be drawn." Thus the example given in the application does not support the claimed activity.

4.42 In remaining paragraphs 4.19-4.24 Dr. Mattick does nothing more than catalogue the uses alleged for VEGF2 in the opposed application. In my first declaration I explained in detail that there is no sound scientific basis upon which these statements of activity were based or would be trusted by a practitioner in this field (OPR1 at 4.6.3.1 and 4.6.5.), and I repeat those explanations here by reference. As I explain above in paragraph 4.13, repeated here by reference, the cataloging of the activities in



a declaration does not make them more believable — there is still no evidence supporting them. This is precisely the scientific double standard that HGS is asking the public to accept in this proceeding. (See paragraphs 2.2-2.7 above, repeated here by reference.)

4.43 AJM1 Paragraph 4.23 provides a good example of the double standard:

“On page 24 (lines 25 to 31) the patent specification identifies uses of truncated versions of VEGF-2 for inactivating the activity of endogenous VEGF-2. It also discloses how such truncated molecules may be used therapeutically as anti-cancer drugs, to prevent inflammation or to treat solid tumour growth, diabetic retinopathy, psoriasis and rheumatoid arthritis (page 25 lines 4 to 13).” (AJM 4.23.)

The opposed application describes no polypeptides that are fragments of the approximately 326 amino acid mature VEGF2, and does not identify any polypeptides that were shown or believed to have VEGF2 inhibitory activity.<sup>10</sup> Nor does the application provide any evidence that VEGF2 is involved in cancer in any way. Yet Dr. Mattick believes that the application discloses how truncated VEGF2 molecules may be used as anti-cancer drugs. Treatment of cancer continues to be one of the most researched and persistent problems of medical science, and has been for decades. There is no evidence in the opposed application to support this assertion. In my opinion, scientists with ordinary skill and experience, or higher, would not believe that fragments of a novel polypeptide could be used to treat cancer just because a

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<sup>10</sup> In paragraph 4.12 Dr. Mattick references “VEGF-C fragments” described in U.S. Patent No. 6,130,071 issued to Alitalo et al. These VEGF-C polypeptides were equated by Dr. Mattick with VEGF2 polypeptides, but of course they are not described in the opposed application and they are active polypeptides, not inhibitory ones.

patent application says so, when there is no disclosure of the activity of the polypeptide and no evidence of involvement of the polypeptides in cancer.

- 4.44 Dr. Hayward says that it is not reasonable to expect an initial characterization of a gene to provide "a full description, supported by detailed experimental proof, of every property and function that the encoded protein possesses." (ANH1 at 4.8) I agree with that position. However, if an inventor is seeking a patent on a gene, an encoded protein, a variety of "active" variants of the protein and gene, and a variety of uses of the protein and gene and variants, and other subject matter, then it is appropriate to ask the patent applicant to provide a working method for expressing and secreting the protein, and to provide evidence of some portion of the properties or functions. It is not appropriate to award the patent if the patent applicant has left for the public to determine how to make and how to use the invention. However, that is precisely the situation here, where the patent application has not shown how to express and secrete VEGF2 or demonstrated any function for VEGF2.

### C3. Experimentation Relating to Inhibition of VEGF2 Activity

- 4.45 I originally discussed the inadequacies of the opposed application with respect to inhibition of VEGF2 activity in OPR1 at 6.6. Generally speaking, knowledge of a biological activity is a prerequisite to designing ways to inhibit the biological activity. Thus, all of the statements in my original declaration relating to biological activity, and all of the additional remarks on that subject above, are relevant to the lack of fair basis and lack of sufficiency regarding claims which pertain to inhibition of VEGF2.
- 4.46 AJM1 4.107 - 4.108. Dr. Mattick's remarks in support of inhibition or antagonism of VEGF2 are repetitious of his remarks relating to VEGF2 biological activity, which I discuss extensively above in Section C2 and repeat here by reference.

### C4. Is VEGF2 a Lymphatic Growth Factor?

4.47 In my first declaration I discuss published evidence (largely from Professor Alitalo's group) that the principle activity of the real-life protein product of the "VEGF2 gene" in healthy mammals appears to be a growth factor for the lymphatic vessels.<sup>11</sup> This fact appears uncontested in HGS's evidence-in-answer.

4.48 It is also uncontroverted that, notwithstanding the numerous activities prophesied for VEGF2 in the opposed application, the inventors failed to teach that VEGF2 was a lymphatic growth factor. The big list of uses and activities that HGS taught, e.g., at page 16 of its application were simply copied from what other scientists had said about VEGF (See Document D36, U.S. Patent No. 5,219,739 beginning at col. 12, line 5 or PDGF.)

4.49 Dr. Mattick attempts to dismiss this shortcoming by drawing an analogy between characteristics of lymphatic vessels and blood vessels, perhaps implying that the opposed application was generic to both when it discusses endothelialization. (AJM1 4.74-4.75) However, he does not contest that the opposed application is completely silent about lymphatic growth or treatment of lymphatic disorders, and it is clear from the context of the alleged VEGF2 uses in the application that they were not contemplating uses relating to lymphatics. Although a bit crude, I would analogize Dr. Mattick's obscuring the distinction between blood vessels and lymphatic vessels to an auto mechanic obscuring the distinction between an auto's fuel lines and its cooling system. Both systems involve vessels (hoses) and liquid flows, but ordinary people in the field appreciate the significant differences in their functions.

#### C5. Experimentation Relating to "Examples"

4.50 In paragraph 4.13.1 of my first declaration, I explain that Example 1 of the opposed application has errors and inconsistencies, the most serious of which is that the results

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<sup>11</sup> I also explain that the opposed application fails to teach the real-life protein product of the VEGF2 gene. [See OPR1 at 4.7.]

which were reported (VEGF2 mRNA of 1.6 kb/kd or 1.3 kb) are discredited by HGS's own subsequent work and work by at least two other independent research groups, which teach that the real mRNA is 2.2-2.4 kb in size. Dr. Mattick spends five paragraphs attempting to rehabilitate Example 1 for HGS (AJM1 4.84-4.88), but he does not contest the fact that HGS's experimental results are just plain wrong. The work reported in Example 1 misinformed the public about the identity and the size of VEGF2 mRNA.

4.51 In paragraph 4.13.2 - 4.13.3 of my first declaration I explain that Example 2 provides information of minimal value to understanding VEGF2 (4.13.2) and that the Example is full of errors and inaccuracies, and is incomplete. (4.13.3) The former is uncontested by Dr. Mattick. With respect to the errors, inaccuracies, and incompleteness, Dr. Mattick spends three paragraphs basically confirming my analysis, admitting that "there are a number of errors" (4.90); that descriptions are "inconsistent" with figures (4.91); that primers are misidentified or not identified at all (4.92); and the like. Dr. Mattick's attempt to rehabilitate the defective Example 2 is just another example of the scientific double standard being applied by HGS's declarants.

4.52 Dr. Hayward spends four paragraphs discussing Example 2 of the opposed application. (ANH1 at 4.26-4.29.) He more or less confirms that Example 2 failed to teach the F5 primer, and that the F4 primer (part of primer pair 2) could not be used to make the polypeptide that was allegedly made. (Dr. Hayward's interpretations of these errors was that F4 as taught in the Example is really F5, and that F4 is missing.) Dr. Hayward declares that he would have been able to carry out experiments similar to those shown in Example 2, but that does not change the plain fact that Example 2 was described inadequately and it would have been left to the reader to experiment simply to try to repeat the "Examples" in the application.

#### C6. Summary Regarding Experimentation

4.53 To summarize, a large percentage of the evidence-in-answer is comprised of explanations of how the declarants, through experimentation, believe that they could have figured out that the opposed application was plain wrong in its characterization of VEGF2. And, after figuring out that the opposed application was wrong, the scientists believe that they could have performed additional experimentation to characterize VEGF2 correctly and then use it for whatever purposes their experimentation revealed VEGF2 could be used for. Thus, the evidence-in-answer appears to be advocating the position that HGS should be awarded a patent because the patent application would have created curiosity and enthusiasm to experiment, because with substantial experimentation one might have discovered the inaccuracies in the patent application, discovered that the invention does not work as described, and discovered alternatives or improvements that might have worked but that were not taught in the application. The question resolves to one of whether it is fair to grant a patent to the person that teaches an incomplete and incorrect invention that others might eventually be able to complete and use. In my opinion this is not fair. The merits of the patent application should be judged on what it teaches, and not how other scientists feel they could have overcome its substantial shortcomings and errors with experimentation. The fact that such experimentation might have revealed the inaccuracies in the opposed application or revealed alternative inventions to the invention described therein does not cure the errors or inadequacies of the application.

**D. INAPPROPRIATE USE OF HINDSIGHT TO MISSTATE THE AMOUNT OF EXPERIMENTATION THAT WOULD HAVE BEEN NEEDED TO MAKE THE INVENTION WORK.**

**D1. Hindsight with respect to VEGF2 Expression.**

4.54 I disagree with much of the evidence-in-answer because it is based on hindsight. In other words, the HGS declarants are drawing conclusions based on what is now known about VEGF2 and VEGF-C, based on papers and patents that were published after the filing date of the patent application. In this section I explain the hindsight

and provide a fairer analysis involving only the common general knowledge in 1994, combined with the teachings in the opposed application.

4.55 Dr. Mattick concludes that, even though VEGF2 as taught in the opposed patent contains only about 350 amino acids, "the nucleotide sequence disclosed in Figure 1 may also be read to encode an additional 23 amino acids." (AJM1 at 3.22 and 4.7.) Dr. Hayward drew a similar conclusion. (ANH1 at 3.14 and 4.18.) As I explain below, Dr. Mattick's and Dr. Hayward's conclusion is contrary to the plain teachings of the opposed application. However, it is easy to see how they would have reached this conclusion when one considers documents published after the opposed application, which taught that VEGF2 includes additional amino acids. That is why their analysis is based on hindsight.

4.55.1 Figure 1 of the opposed application, to which Dr. Mattick and Dr. Hayward refer, depicts a DNA sequence and a deduced VEGF2 protein sequence. The opposed application explains Figure 1 as follows: "Fig. 1 depicts the polynucleotide sequence which encodes for VEGF2, and the corresponding deduced amino acid sequence of the full length VEGF2 polypeptide comprising 350 amino acid residues of which approximately the first 24 amino acids represent the leader." (Opposed Application at p. 4, lines 28-32.) There is no ambiguity in the opposed application as to the length of full length VEGF2 or the location of the methionine amino acid at which full length VEGF2 begins.<sup>12</sup> And there is a clear statement identifying the

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<sup>12</sup> The opposed application explicitly teaches that the methionine codon at the beginning of the VEGF2 protein-encoding sequence is at approximately positions 71-73 of the DNA sequence in Figure 1. The relationship between a polypeptide and the DNA coding sequence encoding it is a relationship of tri-nucleotide codons: each amino acid is encoded by three nucleotide "codon" units. The DNA strands (chromosomes) that characterize the human genome each comprise hundreds or thousands of protein coding sequences, generally

inventor's belief in the division between the 24 amino acid signal peptide and the "mature" VEGF2 of 326 amino acids.

4.55.2 As I explained in my first declaration, multiple groups, including the very inventors of the opposed application, later refuted the notion that the protein encoded by the VEGF2 gene is only about 350 amino acids. It is, in fact, about 419 amino acids, but the opposed application failed to teach about 69 amino acids at the beginning of the protein. [See OPR1 at 4.11.1-4.11.3 (citing Documents D43, D44-46, D84-86 of HGS; D75 and D84 of Genentech; and D71-D74 of University of Helsinki.)]<sup>13</sup>

4.55.3 Thus, it is easy to understand how Dr. Mattick or Dr. Hayward could have used hindsight to conclude that the Figure 1 sequence might actually encode more than 350 amino acids, because numerous publications from about 1996-2001 reported this. But the conclusion is clearly biased on what is now known in year 2001, and contrary to the plain teachings of the opposed application.

4.56 Dr. Mattick compounds this hindsight by opining that he would have learned from the patent application to express VEGF2 with these extra 23 amino acids:

Taking into account the existence of the additional 23 amino acids at the N-terminal end of the VEGF-2 sequence disclosed in the patent specification, I would also have stitched a signal secretion sequence to the

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separated by stretches of non-coding sequences. Each protein coding sequence begins with the codon ATG, which encodes methionine.

<sup>13</sup>Interestingly, the 373 amino acid VEGF2, which has no basis in the application, also has no apparent significance in human biology. The HGS declarants have acknowledged that the full length VEGF2 (with signal sequence) is actually 419 amino acids. The Alitalo research group has shown that the 373 amino acid molecule is vastly different than the forms of protein secreted by human cells.

beginning of the cDNA disclosed in the patent specification, as is taught in the patent specification. I note that such an experiment was done and is described in Australian Patent Application 60467/96 (714,484) (HGS' second VEGF-2 patent application) and the resultant product from those experiments is reported to be biologically active (see page 42, line 32 to page 43 line 25 and Figures 8 and 9 -- HGS' second VEGF-2 patent application). (AJM1 4.8)

- 4.57 The hindsight knowledge of the "additional 23 amino acids" that are not taught in the application is compounded here by a hindsight knowledge gained from HGS's second VEGF2 application to attach a signal peptide to the beginning of the additional 23 amino acids. To the extent the opposed application suggests attaching a foreign signal peptide to a VEGF2 sequence, the suggestion would have been to attach it to "mature VEGF2" which comprised only about 326 amino acids (see opposed application at pp. 4-7). It would have been highly irregular to attempt to attach a signal peptide-encoding sequence to what was taught to be 69 nucleotides of noncoding sequence, because signal sequences are not ordinarily used to drive expression of noncoding sequences. This is especially true when the patent application taught that a true signal sequence immediately followed the 69 nucleotides of noncoding sequence. (Polypeptides don't need two signal peptides for expression and secretion.)

- 4.58 Dr. Mattick devotes three pages to explaining how he would have expressed VEGF2 using "routine trial and experimentation." However, Dr. Mattick has used hindsight in his explanation because his explanation *begins* from the following assumption:

"Had I attempted to express the VEGF-2 protein using the putative secretion signal sequence identified in the patent specification and had that not worked . . . ."  
(AJM1 4.5.)



4.59 Dr. Hayward begins his analysis from a similar vantage point. (See ANH1 at 3.17.) The HGS declarants start here because, with hindsight knowledge, they know that the signal sequence taught in the opposed application does not work. However, at the time the patent application was filed, scientists with ordinary knowledge presumably would not have known that the signal sequence taught in the application would not work. Such scientists would have read the teachings in the application concerning the sequence listing, tried to express the polypeptide according to the application, and failed. Then, the person might have repeated his experiment and modified any of a number of parameters (cell type, expression vector, promoter, growth conditions, and so on) to try to discover why things did not work. Extensive experimentation might have been necessary before arriving at the true source of the problem and solution.

4.60 A similar analysis applies to the manner that most of the other HGS declarants say that they would have successfully expressed and secreted a VEGF2 polypeptide. At least Drs. Gamble, Dr. Hayward, and Dr. Rapoport say that, even though the VEGF2 taught in the patent application is incomplete and the portion of the sequence described as the signal is incorrect, they would have expected to be able to successfully express and secrete VEGF2 by attaching a heterologous (foreign) signal sequence to the 350 amino acids of VEGF2 that were taught in the patent application. (See AJG1 at 6.5; ANH1 at 3.20-3.22; ATR1 at 13.) Dr. Power reported that she performed such an experiment (ASP1), and Dr. Aaronson spoke approvingly of that experiment. (See, e.g., ASA1 at 15-22).

4.61 The notion of expressing the 350 amino acid VEGF2 taught in the opposed application with a heterologous (foreign) signal peptide may be an interesting scientific curiosity, but it has nothing to do with the teachings in the opposed patent application. The opposed application teaches the reader that the VEGF2 of about 350 amino acids *already* consists of a leader sequence (i.e., a signal sequence) representing the first approximately 24 amino acids, and a mature protein of 326 amino acids. (See, e.g., page 5 of opposed application.) Scientists in 1994 or today would not have had any reason to express a protein that already contained a signal sequence using a method that involved attaching a heterologous (foreign) signal

sequence (e.g., Dr. Power's Ig Kappa signal sequence) to the beginning of the natural signal sequence. Such an experiment is not a replication of any example in the opposed application or a reasonable extension of any of its teachings. The instructions that HGS gave to Dr. Power for her experiments were not to replicate the teachings of the opposed application. (See ASP1 at 2.) One might infer that HGS gave these instructions because they knew that the invention taught in the patent application does not work the way that they said it would. (Dr. Alitalo's first declaration (OKA1) showed this.) And the suggestion by the HGS declarants that they would have identified the cause of the failure without extensive experimentation is based on the hindsight knowledge of what they now believe will work.

4.62 The only suggestion to perform the experiment of attaching a heterologous (foreign) signal sequence to the 350 amino acid VEGF2 comes from scientific and patent literature regarding VEGF-C or VEGF2 that was published after the filing date of the opposed application. When one reads that literature, one learns that the gene in actuality has 419 codons, and that the 350 codon sequence was incomplete and lacked a signal peptide. (See OPR1 at, e.g., 1.5.1.1 and 4.11-4.11.1.3; ATR1 at 8.) One also learns (from the VEGF-C literature) which forms of the molecule have biological functions, such as stimulation of the lymphatic system. Even if the experiments involving a heterologous sequence in combination with 350 amino acid VEGF2 work, the results should not be credited to the opposed application, but rather, to the ingenuity of others who determined that VEGF2 does not work in the manner described in the opposed application, but works in a totally different manner.

4.63 Dr. Rapoport's analysis of the signal peptide issue also suffers from hindsight. For example, Dr. Rapoport declares that it was known that signal sequences located at the N-terminus of proteins "were required to directed secreted proteins outside of the cell." (ATR1 at 4-5). He says that researchers were able to characterize such sequences by inspection or with software. (ATR1 at 5-6). Dr. Rapoport says that he fails to observe a typical conserved motif of a signal sequence in the 350 amino acid VEGF2 taught in the opposed application. (ATR1 at 10.) Given what he says about signal sequences in paragraphs 4-6, one might have expected Dr. Rapoport to

conclude that VEGF2 was not secreted at all. (That would have been a logical inference to make about a protein that does not appear to have a signal sequence.)

4.64 Instead, Dr. Rapoport says that there was "strong evidence" that the 350 amino acid VEGF2 was a secreted protein "based on the teaching and recognition of the HGS specification that it is a member of a family of secreted growth factors." (ATR1 at 10 and 7.)

4.65 However, the "strong evidence" upon which Dr. Rapoport relies does not withstand scrutiny. The only "evidence" is the recognition that VEGF2 exhibited 22-30% identity with VEGF and the PDGF proteins. (ATR1 at 7, 9.) I explained in detail in my first declaration that this low level of sequence identity does not permit reliable prediction of structure or function. (See OPR1 at 4.63-4.65.) That portion of my first declaration was not contested by any HGS declarant. There are many examples in the literature of proteins with significantly greater structural similarity, but divergent function. In fact, the examples in my first declaration demonstrate that 22-30% sequence similarity is not even sufficient to be sure that two proteins are both growth factors, or are both secreted from cells. Here are two more examples directly relevant to the field of blood vessel growth:

4.65.1 The angiopoietins comprise a "family" of proteins believed to modulate endothelial cell growth in humans. Although angiopoietins share amino acid identity of, e.g., about 40-70%, some family members appear to stimulate receptors involved in endothelial cell growth, whereas others appear to antagonize (block) them. Thus, angiopoietins share greater sequence similarity with each other than VEGF2 shares with VEGF, but angiopoietins can have directly opposing activities towards each other, with some being growth factors and others being growth factor antagonists.

4.65.2 The fibroblast growth factors (FGF's) comprise another "family" of growth factors. Some, but not all, members of this family have signal peptides for directing secretion of the protein from cells. The FGF's demonstrate that not

all proteins in a particular protein "family" can be expected to have a traditional signal sequence.

4.66 In fact, even if there were sufficient evidence in the opposed application to conclude that VEGF2 were a growth factor, it is untrue that all growth factors are secreted. The ephrins represent yet another "family" of proteins, that like VEGF, have members that appear to play a role in vascular development and interact with their own receptor tyrosine kinases (known as the Eph receptors). All known ephrins are tethered naturally to the cells in which they are expressed, and must normally remain attached to the cell membrane to activate their receptors or other cells. Ephrins B1-B3 all have transmembrane and cytoplasmic domains that localize in the cell's membrane and cytoplasm (interior), respectively.

4.67 The foregoing examples and the uncontested examples from my first declaration demonstrate that Dr. Rapoport had no "strong evidence" upon which he could base his conclusions, except for hindsight knowledge gained from the reported work of HGS's competitors.

4.68 In my opinion, the only true motivation for the research that Dr. Rapoport describes involving foreign signal peptides attached to 350 amino acid VEGF2 comes from knowledge of the 419 amino acid VEGF2 (ATR1 at 8) and published information about VEGF-C (ATR1 at 13-17.) The allegations about what he would have recognized, predicted, and/or expected based solely on the application do not withstand scrutiny.

## D2. Hindsight with respect to VEGF2 biological activity.

4.69 I discuss Dr. Mattick's and Dr. Gamble's hindsight with respect to VEGF2 biological activity above in paragraphs 4.27-4.40 which I repeat here by reference.

4.70 Dr. Hayward also exercises hindsight in his discussions of VEGF2 biological activity. (See, e.g., ANH1 at 3.6; 3.27-3.36.) He explains that he understands VEGF2 activity

to mean one select list of functions that are recited in the opposed application (such as angiogenesis or promoting endothelialisation or wound healing) but fails to list other functions, such as growth of damaged bone, periodontium, or ligament tissue as stated at page 17 of the opposed application. (See, e.g., ANH1 at 3.6; 3.28.)

- 4.71 Dr. Hayward also exercises hindsight by declaring that one of ordinary skill would have considered whether VEGF2 bound to Flt1, Flk1, and Flt4. (See ANH1 at 3.30 - 3.35.) Receptor tyrosine kinases (RTK's) serve as receptors for a large number of molecules, including the PDGF's. My current scientific work includes RTK and RTK inhibitor research, and in my estimation the human genome contains perhaps 500 to 1000 different RTK's (only a small fraction of which would have been characterized in 1994). The suggestion that a person would have focused on only those three receptors (Flt1, Flk1, and Flt4) is based on hindsight knowledge that fully processed VEGF-C was shown by Alitalo et al. to bind to two of them. The idea to screen Flt4 or other particular receptors is not found in the application.

**E. CLAIMS WHICH RECITE PARTICULAR SUBSETS OF THE VEGF2 SEQUENCE LACK FAIR BASIS AND LACK SUFFICIENCY. REPLY TO AJM1 4.96-4.102 AND AJG 6.8.1-6.8.2.**

- 4.72 In paragraph 5.8.1 - 5.8.6 of my declaration I explained that the opposed application as written provided no indication that the applicants intended to claim the particular sequences of claims 11-12, 32-35, and 40-41, and that the only mention of these portions of VEGF2 were not as peptides, but rather, were as observations that they represented portions sharing conserved motifs with the prior art. Dr. Mattick gleans such an intention from identification of the conserved motifs at page 5 and Figure 2, in combination with the teaching at page 9 about "fragments of VEGF-2." I am not persuaded by this analysis.
- 4.73 When I read the discussion of page 9 concerning fragments, I see no mention whatsoever of conserved motifs or of the alleged "signature" sequence. When I read page 5 and Figure 2, I see a discussion of the signature motifs but no mention of

VEGF2 fragments of the invention. I only see an observation that VEGF2 shares two attributes with VEGF and PDGF, and neither attribute is actually a VEGF2 "fragment."<sup>14</sup> Nowhere in the application is the "signature for the PDGF/VEGF family" stated to represent a "VEGF2 fragment" of the invention.

4.74 Dr. Mattick apparently does not dispute my conclusion that claim 31 is entirely unsupported in the application. (See OPR1 at 5.8.7), or my observations in paragraph 5.8.9 regarding failure to adequately teach VEGF2 "activity" information about these peptides, even if they were adequately described.

4.75 In paragraph 6.8.1 - 6.8.2 Dr. Gamble summarily states that she believes that the patent specification provides "a range of different length VEGF-2 polypeptides." as well as the DNAs encoding them. For reasons explained in my first declaration and in the preceding paragraphs, I disagree.

F. LACK OF FAIR BASIS OR SUFFICIENCY FOR VEGF2 MRNA.

4.76 In AJM1 4.103 - 4.104, Dr. Mattick responds to my criticism in OPR 5.8.10 that the opposed application lacks support for VEGF2 mRNA because the alleged mRNA in the opposed application is clearly misidentified. Dr. Mattick cites Example 1 as providing support for VEGF2 mRNA. Dr. Hayward provides analysis of Example 1 at ANH1 paragraphs 4.21-4.24.

4.77 I am unpersuaded by Dr. Mattick's and Dr. Hayward's analysis. I note apologetically that OPR 5.8.10 should have referred to Example 1 in the opposed application, rather than Example 2. A review of Example 1 proves my original point. In paragraph 4.13.1 of my first declaration, I explained that Example 1 of the opposed application

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<sup>14</sup> What I mean by this is that the first attribute - eight conserved cysteines - are scattered throughout the VEGF2 sequence, not a fragment of it. The second attribute is a generic chemical formula PXC VXXXR C XGCCN, not a VEGF2 fragment.

has errors and inconsistencies, the most serious of which is that the results which were reported (VEGF2 mRNA of 1.6 kb/kd or 1.3 kb) are *discredited by HGS's own subsequent work and work by at least two other independent research groups*, which teach that the real mRNA is 2.2-2.4 kb in size. Dr. Mattick relies on Example 1 and spends many paragraphs attempting to rehabilitate Example 1 for HGS (AJM1 4.84-4.88, 4.104), but he does not contest the fact that HGS's experimental results are *just plain wrong*. The work reported in Example 1 misinformed the public about the identity and the size of VEGF2 mRNA.

- 4.78 Dr. Hayward also does not contest that the 1.6 kb/kd result reported in Example 1 and relied upon by the HGS declarants in various aspects of their declaration is wrong, and discredited by subsequent experiments. However, Dr. Hayward attempts to rehabilitate Example 1 by interpreting a different experimental result, namely Figure 5. Dr. Hayward says that he would judge Figure 5 as showing a band of about 2.4/2.3 kb and a band of 1.6 to 1.8 kb and that "at least the major band is reasonably consistent with subsequent results." (ANH1 4.24).
- 4.79 Whatever meaning Dr. Hayward can now discern from Figure 5 does not change the fact that HGS attributed no significance to Figure 5 whatsoever when it wrote the opposed application. Nor does his interpretation change the fact that the result upon which the inventors told the public to focus, 1.6 kb/kD, is now discredited.
- 4.80 Moreover, it's not clear to me that Dr. Hayward's interpretation of Figure 5 is even reliable. He admits that there is at least one mislabeled band (ANH1 at 4.24-4.25) and it appears to me that there may be more than one mislabeled band. There is no basis in the application to determine which of the band inconsistencies in Figure 5 are right or wrong (or whether all are wrong).

G. THE PATENT APPLICATION MIS-TAUGHT VEGF2 PROTEOLYTIC PROCESSING.

- 4.81 Dr. Gamble devotes several pages of her declaration discussing knowledge of prior art growth factors PDGFs, VEGF, and PlGF, including discussion of proteolytic processing of these proteins. (See AJG1 5.1-5.13.) Her apparent reason for the lengthy discussion of the prior art is an attempt to rehabilitate the teachings of the opposed application relating to VEGF2. (See AJG1 6.1 - 6.5; 7.23-7.24.) Dr. Rapoport's declaration also relies heavily on analogy to VEGF and PDGFs processing for his declaration. (See, e.g., ATR1 at 7, 9, and 11.)
- 4.82 First, it is important to remember that the opposed application taught an incomplete VEGF2 sequence, missing about 69 codons/amino acids. At least three of HGS's declarants have admitted this important omission from the patent application. (See paragraphs 4.3-4.3.4 above, repeated by reference.)
- 4.83 When assessing the sufficiency of the opposed application, it is also important to bear in mind its actual teachings. The opposed application taught the inventors' best prediction at the time regarding proteolytic processing (i.e., signal peptide of 24 amino acids, mature protein of 326 amino acids), which was plain wrong. Dr. Alitalo's two declarations confirm that cells cannot express and secrete VEGF2 taught in the patent application. (See Dr. Alitalo's declarations, OKA1 and OKA2.)
- 4.84 Knowledge of the proteolytic processing of prior art proteins, discussed extensively by many of the HGS declarants, does not support patentability. If anything, it negates it. The HGS inventors used knowledge of prior art proteins (e.g., VEGF) to predict the signal peptide and mature portions of VEGF2 in their patent application, which is essentially what the HGS declarants say that they would have done. (See AJG1 6.5: "Thus, I would predict that VEGF-2 would be expressed in a similar way." See also AJG1 6.10 - 6.11; ANH1 at 3.15; ATR1 at 11: "I would predict VEGF-2 to be expressed in a similar way [as the PDGF/VEGF family of growth factors'.") HGS was plain wrong in the opposed application, and HGS's declarants likewise would have been wrong in their prediction. The processing taught by the opposed application (simple removal of a signal peptide at position 1-24) does not occur. (See OKA1 and OKA2.)



4.85 Moreover, the full length 419 amino acid protein (VEGF-C) is processed in a manner unexpectedly different and more complex, compared to simple removal of a signal peptide. (See OPR1 at 4.11.1.3 and Documents D70, D71, D73, and D74 cited therein.) Therefore, I must disagree with Dr. Gamble's suggestion that "VEGF-2 is no different in that it contains a signal or leader sequence." (See AJG1 at 6.4; see also AJG1 at 6.3) The processing of the full length VEGF-C protein is substantially different. Even when HGS discovered the 419 amino acid VEGF2 sequence and filed its second patent application, they still badly mis-predicted the manner in which the protein is processed.<sup>15</sup>

4.86 Thus, the only relevant portion of Dr. Gamble's declaration or the other declarations relating to proteolytic processing is her admission that the opposed application was incomplete. She plainly agrees that the VEGF2 signal/leader sequence "is located among the 69 amino acids that were not disclosed in the patent specification." (AJG1 6.4 (emphasis added).)

4.87 In paragraph 7.24, Dr. Gamble declared as follows:

I was aware that all members of the PDGF/VEGF family (known in March 1994) underwent some proteolytic processing when produced from a cell. Thus, had I been asked to examine VEGF-2 in March 1994 I would have expected that VEGF-2 may also naturally undergo some proteolytic processing when released from a cell. This process occurs naturally during secretion, controlled by cellular enzymes. Consequently, if a

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<sup>15</sup> In its second generation VEGF2 patent applications, Human Genome Sciences taught that 419 residue VEGF2 has putative leader sequence of approximately 23 residues such that the mature protein comprises 396 amino acids (See Document: D43 and D44 at p. 7, last paragraph). In fact, the leader is 31 amino acids, and the mature protein is much smaller due to additional proteolytic processing. See Document D71.

researcher were able to produce VEGF-2 and secrete it from a cell I believe a reasonable expectation would be that processing would take place. Therefore, in my opinion, the patent specification fully enables one to express and secrete a biologically active protein that has been correctly processed.

- 4.88 Dr. Gamble's qualifier "if a researcher were able to produce VEGF2 and secrete it from a cell" cannot be overemphasized, because Dr. Alitalo has shown that VEGF2 as taught in the patent application is not expressed and secreted. Her statements about "reasonable expectation" are really "pure speculation and cannot reasonably be drawn" (see AJG1 7.52.)

H. THE FACT THAT HGS's DECLARANTS WOULD HAVE MADE THE SAME MISTAKES AS THE HGS INVENTORS DOES NOT REHABILITATE THE OPPOSED APPLICATION

- 4.89 Dr. Aaronson briefly explains that, had he been presented with a novel 350 amino acid VEGF2 sequence in March 1994, he would have (a) predicted that it was proteolytically processed; and (b) predicted that it was biologically active. (See ASA1 at 5 - 6.) Dr. Gamble makes similar predictions. (See AJG1 at 6.5.) In my opinion, what should matter is whether or not the application is correct, and not whether Dr. Aaronson or anyone else would have made favorable or unfavorable "predictions."
- 4.90 Processing: As I discuss in detail in the preceding section, the HGS scientists also predicted proteolytic processing in a manner analogous to VEGF. (See paragraphs 4.71-4.75.) The important point is that they were wrong, and what they taught the public in the opposed application was wrong. First, the VEGF2 was incomplete, and is not processed by cells in the way that Dr. Aaronson or the HGS inventors predicted. Second, even when the full 419 amino acid VEGF2 was discovered by the HGS inventors much later, they still predicted wrong. Dr. Alitalo's science has shown that the proteolytic processing of VEGF-C is much more complicated than the proteolytic processing that occurs with VEGF. See Document D71.

- 4.91 Activity: There is still no evidence in the patent application or the ensuing literature of the next seven years that has been made of record in this proceeding that the VEGF2 taught in the *opposed application* is "biologically active." HGS's declarants believe that the requisite testing for activity would have been "routine" (see, e.g., ANH1 at 4.7), but HGS has only reported activity data for molecules that were taught in its *second generation* application or molecules taught by *other research groups* (e.g., Alitalo et al. for VEGF-C) that differ from VEGF2 of the opposed application.

I. THE EVIDENCE OFFERED BY HGS IN SUPPORT OF THE OPPOSED APPLICATION RELATES TO A DIFFERENT INVENTION THAN THE ONE TAUGHT IN THE APPLICATION.

- 4.92 In this section I explain that the important data and opinions offered by HGS in its evidence in answer does not actually relate to the invention of the opposed application. Rather, it relates to subsequent inventions.
- 4.93 The teachings in the opposed application concerning full length and mature VEGF2 are unequivocal: "The polynucleotide of the invention . . . contains an open reading from encoding a protein of about 350 amino acid residues of which approximately the first 24 amino acid residues are likely to be leader sequence such that the mature protein comprises 326 amino acids . . . ."
- 4.94 Notwithstanding these clear teachings, HGS has devoted a vast portion of its declaration evidence arguing about the merits of totally different VEGF2 proteins that were not taught in the application.
- 4.95 For example, HGS asked Dr. Power to treat the 350 amino acid VEGF2 as "mature VEGF2" and try to express it by attaching a different signal sequence to it: "The Patent Attorneys for Human Genome Sciences (HGS) requested that I perform the following experiments in order to determine whether the 350 amino acid form of VEGF-2 (corresponding to residues 70 to 419 of the 419 amino acid form of VEGF-

2) fused in frame with a heterologous signal sequence would result in the expression and secretion of the protein from eukaryotic cells.” (ASP1 at 2.)

- 4.96 Dr. Aaronson cited Dr. Powers work expressing a 350 amino acid VEGF2 with a different signal sequence with approval. (See ASA1 at 15 - 22.)<sup>16</sup> Dr. Gamble endorsed the same approach for “attempting to express” the VEGF2 sequence which she noted, with hindsight, was missing the real VEGF2 signal sequence. (AJG1 6:4-6.5.)
- 4.97 Dr. Mattick was even more creative in declaring that he would have been motivated to express a 373 amino acid VEGF2 with a different signal peptide. (See discussion herein at paragraphs 4.19-4.20.) Dr. Hayward came down somewhere in between, because he agreed with Dr. Mattick that he could find 373 amino acids supported in the application, but he then discusses how he might have attempted to express the 350 amino acid VEGF2. (ANH1 3:13-3.26)
- 4.98 These hypothetical experiments (real experiments in the case of Dr. Power) may be interesting to consider, but they have nothing to do with what the opposed application taught the public, because it taught the public that mature VEGF2 was 326 amino acids.
- 4.99 One explanation given by the HGS experts for their creative approaches was that the opposed application taught that one could express VEGF2 using a heterologous leader sequence. See ASA1 at 16; AJM1 at 4.9. However, a scientist of ordinary skill who desires to express a secreted protein using a heterologous leader sequence will use the heterologous leader to *replace* the native leader sequence. (For example, replace amino acids 1-24 of VEGF2 with a heterologous leader sequence, attached to

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<sup>16</sup> Dr. Aaronson declared that Dr. Power succeeded in producing “a biologically active” protein. (See ASA1 17.) I dispute this characterization. Dr. Power did not report any activity assay data in her declaration. She reported only an expression study.

positions 25-350.) There is nothing in the opposed application teaching that one should substitute a heterologous leader to the 350 amino acid VEGF2.

- 4.100 The inference that could be drawn from all of this is that neither HGS (who designed Dr. Power's experiments) nor its declarants believe in the 326 amino acid mature VEGF2 taught by the opposed application.
- 4.101 Dr. Hayward declares that he is aware that VEGF2 is proteolytically processed upon secretion from cells in vivo to form the naturally occurring ligand for the Flt4 and KDR/Flk-1 receptors. (ANH1 at 3.22. See also ANH1 at 3.30- 3.35.) He is mistaken. As I explain in my original declaration (OPR1 at 2.7.18) and again above (e.g., Paragraph 2.15), fully processed VEGF-C, invented and patented by Alitalo et al. (see, e.g., U.S. Patent Nos. 6,221,839 and 6,245,530, Exhibit PAWR-13 and Exhibit PAWR-14, respectively), is a ligand for Flt4 and KDR/Flk-1. The 350 amino acid VEGF2 as taught in the original application was not taught to be a ligand for any receptor, cannot be expressed and secreted by cells, and has never been reported in the literature to be a ligand for these receptors.
- 4.102 Dr. Hayward also declares that VEGF-2 "of course" functions as a growth factor. This, too, has never been established. The VEGF-C forms invented by Professor Alitalo's group have been reported to have certain growth factor activities, and the HGS II application alleges that some unidentified form of VEGF2 may have exhibited some growth factor activity in some experiments. However, the VEGF-C polypeptides of Alitalo and the 419 amino acid VEGF2 from the HGS II application are not the teachings of the opposed application.
- 4.103 Dr. Hayward also declares, "page 4 lines 12 to 14 of the patent specification states that the VEGF-2 polypeptides of the invention may be used to isolate receptors of VEGF-2. At page 24 fifth paragraph to page 25 first paragraph the patent specification discloses that VEGF-2 binds to tyrosine kinase receptors on the surface of target cells to activate endothelial cell growth." (ANH1 at 3.30.) This statement falsely attributes the teachings of Alitalo et al. to the inventors of the opposed.

application. The opposed application contains no teaching whatsoever of the identity of VEGF2 receptors, but only speculation that there are such receptors. More than a year later, when HGS filed its second series of applications on the 419 amino acid VEGF2, HGS still had failed to report the identity of any VEGF2 receptor. (See Documents D44-D46.)

J. MISCELLANEOUS SPECIFIC COMMENTS TO EVIDENCE-IN-ANSWER.

4.104 In this section I try to address any remaining parts of the HGS declarations that appear to relate to issues of fair basis and sufficiency.

J1. AJM1 4.61-4.68

4.105 In paragraphs 4.61-4.68 Dr. Mattick spends about two pages attempting to rebut my explanation that the opposed application fails to teach or exemplify any *active* fragments, analogs, or derivatives of VEGF-2, or any such fragments, analogs, or derivatives with *inhibitory* activity.

4.106 Dr. Mattick first cites to Example 2 as allegedly describing "a fragment." (AJM1 4.63) Even if one fragment is described there, the application provides no evidence or indication that it is "active" or is an "inhibitor". Moreover, as I explain in my first declaration, the description of this example is defective. (See OPR1 at 2.7.20 and 6.6.)

4.107 He also cites an excerpt from page 5 of the opposed application. (AJM1 4.63.) As I explain in my first declaration, this excerpt is nothing more than the inventors attempting to characterize features of the complete VEGF2 molecule - there is no teaching there of fragments. (OPR1 at 3.4.2.) Moreover, even if these were fragments, there is again no teaching or evidence of activity or inhibitory properties.

4.108 Dr. Mattick then launches into a discussion of the experimentation that he could have done to make fragments and then test them for activity or inhibitory properties. This discussion of what Dr. Mattick might have been able to do "to identify" such

molecules only proves my original point that the application itself fails to teach them. His explanation of a research plan for arriving at active fragments, analogs, or derivatives, or inhibitory ones, would be unnecessary if the application had described any active fragments to which he could refer.

J2. AJM1 4.81-4.83

- 4.109 In paragraphs 4.10-4.10.6 of my first declaration I explained that claims in the opposed application defining polypeptides by the ability of antibodies to bind VEGF2 lack any basis in the application. For example, I explained that the application gives no indication that the applicants even intended to define an invention in this manner. (OPR1 4.10.1) Dr. Mattick says he disagrees, but for support, he says that certain pages of the application "provide a variety of suggested uses for which the defined fragments may be used." (AJM1 4.81) Dr. Mattick also urges that he could have made antibodies to the VEGF2 taught in the opposed application. (AJM1 4.82-4.83) He misses the point. Irrespective of whether there would be a use for the polypeptides, the application itself is not directed to such polypeptides. Rather, they appear to be afterthoughts, added to the application in an attempt to cover subject matter invented after the opposed application was filed, such as "full length" VEGF2 on which HGS filed a second application much later, and biologically active VEGF-C polypeptides that were invented by other scientists. (See OPR1 at 4.10-4.10.6.)

J3. AJG1 6.1-6.2

- 4.110 Dr. Gamble credits HGS with succeeding in identifying a VEGF2 gene and polypeptide sequence. (AJG1 6.1) This is inaccurate, in that the proposed application taught an incomplete sequence that cannot be expressed and secreted. When HGS felt that they had identified the complete VEGF2 gene, they filed a second series of VEGF2 patent applications. See discussion in OPR1 at 1.5.1-1.5.1.2 and Documents D44-D46.
- 4.111 Dr. Gamble says that she was aware that the cysteine residues enable the PDGF/VEGF family of proteins to form dimers. (AJG1 6.2) The published evidence

that I have seen suggests that fully processed VEGF-C is different from other members of the family in that cysteines are not involved in forming cysteine-linked dimers. (See Document D71 at p. 3907.)

J4. AJG1 7.15

- 4.112 Dr. Gamble alleges that "The patent specification clearly identifies that VEGF-2 has angiogenic activities, and describes in vitro angiogenic assays of VEGF-2, which could be routinely used." I disagree. There is no description of in vitro angiogenic assays in the patent application. And, to the extent the patent specification "clearly identifies" angiogenic activities, it is only by assertion, unsupported by evidence. In this manner, the patent application also "clearly identifies" many other "activities" such as "to induce the growth of damaged bone, periodontium, or ligament tissues" or to promote the formation of cementum with collagen fiber ingrowths. (See opposed application at p. 17.)

J5. ANH1 3.4

- 4.113 Dr. Hayward declared that "the term VEGF-2 conveys to me the idea of not just the sequences presented in the patent specification, but a family of related sequences. Once a protein has been identified any given sequence can be tested to determine whether any amino acid changes affect the structural and or functional characteristics of that protein." Assuming Dr. Hayward's statements are true in a general sense, they do not apply to VEGF2 taught in the opposed patent application. One cannot test to determine whether amino acid changes affect the structural or functional characteristics of VEGF2 taught in the patent application, because the application does not demonstrate a VEGF2 function, and because VEGF2 taught in the patent application is not expressed and secreted as the application taught it could be.

J6. ANH1 3.37-3.37.5

- 4.114 Dr. Hayward Declared as follows:

3.37 Associate Professor Rogers has commented that fragments of polypeptides have no practical utility whatsoever (see for example paragraph 4.10.2,



Associate Professor Rogers' Statutory Declaration).

Clearly this is not the case. As asserted in the patent specification, fragments of polypeptides can be used to make antibodies, which are useful both experimentally and therapeutically. In addition, active fragments of polypeptides are often used in therapy as antagonists since they can compete with the full length version but may lack full biological activity. Further, such polypeptide fragments could also be used as agonists that might mimic some of the biological activities of the full-length protein. Although the skilled person would be aware of this in any case, I note that the specification discloses the following:

(Dr. Hayward then continues with a discussion of the alleged teachings in the opposed application.)

- 4.115 Dr. Hayward has misstated what I said in OPR 4.10.2. Paragraph 4.10.2 made no sweeping statements whatsoever applicable to the utility of fragments of all polypeptides. In fact, it made no statement whatsoever about fragments of VEGF2 per se. In that paragraph, I explained that claims 16-27, 40-50, and 57-61 contained no limitations relating to biological activity, and that the specification of the opposed application failed to provide an activity commensurate with the genus being claimed. As I had explained in 4.10.2 and elsewhere in OPR1, the literal scope of those claims encompasses more than just fragments of VEGF2 that might have utility.
- 4.116 In addition to misunderstanding 4.10.2, Dr. Hayward expresses ideas that require comment.
- 4.117 For example, he says that fragments of VEGF2 can be used to make antibodies which are useful therapeutically. Since the application fails to demonstrate any biological effect of VEGF2, it is purely speculative to say that antibodies to VEGF2 could be

therapeutic. If someone develops such a therapy, it would owe nothing to the teachings in the opposed application.

4.118 Dr. Hayward also declares that VEGF2 antibodies can be used to measure elevated levels of VEGF2 in individuals, and speculates that such levels may be associated with cancer. Again, there is no evidence supporting this speculation in the patent application. If someone discovers such an association and develops a working diagnostic, that development would be due to independent research, and not to the teachings of the patent application. The VEGF2 taught in the patent application cannot be expressed or secreted by cells, as demonstrated by Dr. Alitalo. Thus, the notion of elevated levels of the VEGF2 *taught in the opposed application* has thus been refuted.

4.119 Dr. Hayward says fragments are often used in therapy as antagonists since they can compete with the full length version but may lack full biological activity. Again, this is merely speculative, since the patent application fails to demonstrate that the full length VEGF2 has any biological function. In fact, the Alitalo declarations persuasively demonstrate that cells cannot even express and secrete full length VEGF2, as taught in the opposed application, so the notion that there is "the full length version" that needs antagonizing lacks credibility.

4.120 Dr. Hayward says some fragments may be used as agonists because they "might mimic some of the biological activities of the full-length protein." As I have stated previously, the whole notion of biological activity or activities of full length VEGF2, taught in the application, is speculative. The notion that fragments could have been made and tested for activities that have not even been demonstrated for full length VEGF2 is even more speculative. If such fragments were found, it would be through the efforts of further research, and not be attributable to the teachings in the application.

4.121 Dr. Hayward also declares that the application "clearly demonstrates" that VEGF-2 is expressed at higher levels in cells derived from malignant tumors. I addressed the

apparent errors in the relevant HGS experiment in paragraphs 4.50-4.52, which I repeat by reference.

J7. ANH1 4.10

4.122 Dr. Hayward declared that the process of determining which parts of a polypeptide molecule are required for activity was routine by 1994. If this were true, it presupposes at least that the activity of the polypeptide was known to begin with. The experimentation that Dr. Hayward is describing could only have been performed after the extensive experimentation that would have been required to successfully express and secrete VEGF2, and the additional experimentation that would have been required to identify its activity (if any), *and* the additional experimentation to make the mutant forms of VEGF2. This experimentation is extensive and unpredictable, and should not be credited to the opposed application, which taught only an incomplete DNA/protein sequence with no proven activity.

4.123 Dr. Hayward declares that the specification highlights eight cysteines and a 14 amino acid signature motif thought to be involved in VEGF-2 function. The specification identifies these areas as a feature shared with the prior art, but provides little guidance otherwise.

J8. ANH1 4.15

4.124 In paragraph 5.7.1 of my first declaration I explained that the opposed application does not indicate an intent to claim a genus of polypeptides that bind an antibody that binds VEGF-2. Dr. Hayward says he finds such an intention at page 23, lines 5-14. (ANH1 at 4.15.) I have reviewed the application again but disagree with Dr. Hayward. Pages 22-24 relate to alleged methods of making and using antibodies, as Dr. Hayward later alleges himself at ANH1 4.16. Page 23 does not indicate to the reader an intention to define a genus of VEGF2 polypeptides.

LACK OF CLARITY

5.1 In my first declaration I explained that claims 51 and 54 and claims dependent therefrom are confusing in that claim 51 seems to require administration of a polypeptide, but dependent claim 54 seems to say that administration of the polypeptide encompasses the act of administering DNA, an activity that does not involve administration of any polypeptide whatsoever. (OPR1 6.8.2) Dr. Mattick confirms my analysis that these claims suffer from this ambiguity by reading them to permit the limitation "administration . . . of the polypeptide" without administering any polypeptide. (AJM1 at 4.109 - 4.112.)

5.2 Indefiniteness of "fragment, analogue, or derivative" claims.

5.2.1 The declarations provided by HGS provide interpretations of "fragment, analog, or derivative" language that are intended to save the claims from encompassing the prior art. However, those claim interpretations, if adopted, raise substantial issues of vagueness which I explain above in paragraphs 3.18-3.25.4, and repeat hear by reference. Even if the standard that they proposed were understandable, it would result in the scope of the claims changing with time, as new genes are discovered.

5.2.2 Dr. Gamble offered the following interpretation of "fragment, analogue, or derivative" in her declaration:

Reference is made on page 9, line 14 to page 10, line 5 of the specification to the meaning of the terms "fragment", "derivative" and "analog". When I read these passages I understood HGS to be saying that a fragment, derivative or analogue is a polypeptide that retains essentially the same biological function or activity as VEGF-2. (See AJG1 7.7 and 7.8.)

5.2.3 I agree with Dr. Gamble that there is some support at page 9 of the opposed application for this interpretation. However, I also observe that claim 56 is directed to a fragment, analogue, or derivative having an inhibitory activity. Dr. Mattick reads other portions of the application as suggesting that VEGF2

fragments can have VEGF2 inhibitory activity. (See AJM1 4.23.) These claim interpretations are irreconcilable.

- 5.2.4 Dr. Gamble also offers the following additional guidance about the definition of fragment, analogue, or derivative:

While I acknowledge this basic requirement I am conscious of the fact that before something can be an analogue, fragment or a derivative of a protein it needs to share sufficient identity with that protein to make it resemble that protein or at least part thereof. In my opinion both of these requirements must be satisfied before a sequence of amino acids can truly be called an analogue, fragment or a derivative of VEGF-2. (AJG1 at 7.7)

- 5.2.5 As I explain in my original declaration and also above, neither the application nor conventional scientific usage clarifies in any meaningful way what "sufficient identity . . . to make it resemble" means. (See OPR1 at 4.6.3; see above at 3.23-3.24.) Even if the concept were understandable, the boundaries are not.

### 5.3 Indefiniteness of "VEGF2 activity."

As I explained in my first declaration, the opposed application contains no explicit definition of VEGF2 activity (OPR1 at 2.3.1 - 2.3.2.3), but suggests many in vivo or in vitro uses for VEGF2 (all unsupported by evidence). (OPR1 at 2.3.3.) I explained in OPR1 that "VEGF2 activity" and related terms were indefinite. (See OPR1 at 6.5-6.5.1, 2.3-2.3.5, and 4.6-4.8.) HGS's declarations cast still further uncertainty on the meaning of VEGF2 activity, because they are ambiguous about whether "VEGF2 activity" means a single parameter or multiple parameters. If a polypeptide must have "all" of these biological activities, it would be impossible to ever complete the tests to determine them. If "one or more" of these activities is sufficient for "VEGF2

activity", then prior art polypeptides have VEGF2 activity. (See OPR1 at 2.3.) If it is not necessary for the polypeptide to retain all of the supposed VEGF2 in vivo and in vitro properties, it is unclear how many such properties can be "lost" before the polypeptide is no longer considered to have VEGF2 activity.

5.3.1 Dr. Gamble declares that she immediately understands biological function or activity "to include at least in vivo and/or in vitro activity." (AJG1 at 7.8) The implication of this statement is that she thinks every use mentioned in the patent application, from culturing vascular endothelial cells, providing cancer therapy, promoting bone or periodontium or ligament growth, etc. is VEGF2 activity. It is still ambiguous whether each alone is VEGF2 activity or whether, together, they comprise VEGF2 activity.

5.3.2 Other parts of Dr. Gamble's declaration suggest that a single biological function is sufficient for "VEGF2 activity." For example, in paragraphs 7.9 and 7.10, and again in paragraphs 7.15-7.18, she seems to be suggesting that testing for angiogenic activity alone is a sufficient test for VEGF2 activity.

5.3.3 Still other parts of Dr. Gamble's declaration suggest that a single biological function is NOT sufficient for "VEGF2 activity." For example, in AJG1 7.11, Dr. Gamble says that whether or not a polypeptide promotes *endothelial cell growth* is a means for distinguishing a polypeptide that has VEGF2 activity from a PDGFa or PDGFb polypeptide, which she says do not promote growth of endothelial cells. Here, she seems to be saying that all VEGF2 polypeptides have at least endothelial cell growth activity (because that is how they can be distinguished from PDGF polypeptides). This endothelial growth activity must be in addition to the "angiogenic" activity she says she could have easily screened for (in AJG1 7.9-7.10 and 7.15-7.18), because Dr. Gamble teaches that PDGF polypeptides also have angiogenic activity. (See Litwin, Gamble, and Vadas, Annexure GBC-10 of the HGS evidence, at page 105 (PDGF "has roles in wound healing and angiogenesis").)

5.3.4 Dr. Hayward says that VEGF2 activity means "one or more" of a list of about seven activities that he selected from the patent application. (See ANH1 3.6) His list explicitly *omitted* many other functions also taught in the patent application, such as growth of damaged bone, periodontium, or ligament tissue as stated at page 17 of the opposed application. It is not clear whether Dr. Hayward now classifies one or more of these other functions as VEGF2 activities. (At paragraph 4.4, Dr. Hayward makes generic reference to all of the activities recited at pages 4 and 16-18 of the application.) Dr. Hayward also is of the opinion that whether the specification teaches a unique defining activity for VEGF2 is irrelevant. (ANH1 at 4.3.)

5.3.5 Thus, even when HGS's declarants are trying to explain the alleged simplicity of understanding and screening for VEGF2 activity, they create more questions than answers. If VEGF2 means "angiogenic activity" then why did the application teach that VEGF2 did so many other things, and how does "VEGF2 activity" distinguish the angiogenic PDGF and VEGF polypeptides of the prior art? If "VEGF2 activity" means the combination of "endothelial growth activity" AND "angiogenesis activity" (per Dr. Gamble, to exclude PDGF from the claims), then why does Dr. Gamble suggest in other paragraphs of her declaration that the only testing she would have needed to do was testing for "angiogenic activity"? And how does this definition of "VEGF2 activity" exclude prior art VEGF polypeptides, which promote endothelial cell growth and angiogenesis? If the combination of "angiogenic activity" and "endothelial growth activity" are the two functions that HGS says are the critical ones for defining "VEGF2 activity", then why does the application identify so many other functions, and why do the experts broadly say that "VEGF2 activity is "in vitro and/or in vivo" activity? And why (except perhaps for hindsight) did the HGS declarants select angiogenic activity and/or endothelial cell growth activity as the two important activities, to the exclusion of all of the other functions mentioned in the application? If a protein induces the growth of damaged bone, periodontium, or ligament tissue as stated at page 17 of the opposed application, does that protein have VEGF2

activity? Does the answer depend on whether it also promotes angiogenesis and endothelial cell growth? For all of these reasons, I find that the declarations of Dr. Gamble and others only confuse further the meaning of "VEGF2 activity."

- 5.3.6 HGS's continued assertion that VEGF2 activity includes antigenic activity (the ability to cause antibody production) raises still more questions. (See paragraphs 3.32-3.35, repeated here by reference.)

5.4 Indefiniteness of "polypeptide binds an antibody which binds to VEGF-2"

- 5.4.1 At least claims 16-22 and 40-50 of the opposed application recite, or depend from claims that recite, the limitation "polypeptide binds an antibody which binds to VEGF-2" or some close variation thereof.

- 5.4.2 HGS's declarants ask the Patent Office to believe that antibody cross-reactivity is simply too unpredictable to draw any conclusions from looking at protein sequences. Exemplary excerpts from the HGS evidence follows:

Associate Professor Rogers appears to suggest (see, for example paragraph 2.7.16) that antibodies that bind to regions of VEGF-2 that are conserved with other PDGF/VEGF family members might be cross-reactive. Associate Professor Rogers refers to a number of prior art disclosures of anti-VEGF or anti-PDGF antibodies. Simply because two sequences share some homology does not necessarily imply that any antibody produced against one molecule would necessarily be cross-reactive against the other. In my opinion no conclusion can be made as to whether sequences sharing some homology will generate antibodies that are cross reactive. (AJG1 7.21)



The fact that there is some sequence homology between VEGF-2 and VEGF and other proteins does not mean that there is a high probability that antibodies to VEGF-2 will cross react with VEGF or those other proteins.

Antibodies generally have exquisite specificity and will only cross react with closely related proteins. I do not believe that any conclusion can be drawn about cross-reactive antibodies at a theoretical or practical level.

(AJM1 at 4.33)

5.4.3 If the Patent Office accepts the premise of HGS's experts, then I believe it should also find that all of the relevant claims which attempt to define subject matter with an antibody binding limitation are indefinite. If no predictions can be made, then HGS is saying that the public is required to physically screen a polypeptide with every antibody that anyone could ever generate against VEGF-2, in order to determine whether the polypeptide falls within a claim. That is clearly an impossible task. The boundaries of the claims are still further obscured because the cutoff level of binding is unspecified.

5.4.4 When criticizing the Alitalo declaration, HGS's declarants ask the Patent Office to believe that the results of an antibody binding experiment can be manipulated to achieve any desired result, by varying such parameters as antibody chosen (because antibodies have different binding affinities); selection of polyclonal versus monoclonal antibodies; the amount of antibodies used; and the lack of established controls. (See, e.g., ANH1 at 5.7.) If the Patent Office accepts HGS's position on this issue, then logically, it must also conclude that all of the claims that define a genus of polypeptides by whether they bind an antibody that binds VEGF2 are unclear. The essence of what HGS is saying is that whether an antibody binds to a VEGF2 polypeptide can be manipulated to achieve a desired result, unless a number of parameters are carefully defined. None of those parameters are defined in the opposed application or the claims at issue. In fact, the entire notion of VEGF2

antibody binding is theoretical in the opposed application, since no antibodies are taught. If antibody binding is subject to all of the variables and manipulations identified by HGS, then the metes and bounds of the claims are also unpredictably variable and subject to manipulation, and cannot be considered clear.

#### 5.5 Indefiniteness of "hybridisation" language in the claims.

- 5.5.1 I explained that hybridization language in the claims was confusing in my original declaration. (See, e.g., OPR1 at 6.7 - 6.7.2.)
- 5.5.2 Dr. Hayward discusses hybridization claim language at ANH1 3.9-3.12. He says that, "When I read the word 'hybridise' in those claims I understand it to mean that the hybridisation reaction should be conducted under suitably stringent conditions such that only VEGF-2 polynucleotide sequences would bind either nucleotide sequence shown in the sequence listing (SEQ ID No. 1) or the cDNA deposited in the ATCC deposit identified in the patent specification or fragments thereof." Needless to say, Dr. Hayward's definition is totally circular. He says he would define the VEGF2 subject matter of the claims as encompassing VEGF2 subject matter.
- 5.5.3 To the extent that Dr. Hayward believes that "VEGF2" can include variants of the precise VEGF2 sequence taught in the application, such as non-human "orthologs" (suggested in ANH1 at 3.3), the application gives no guidance as to how dissimilar such sequences are or what hybridization conditions would be appropriate for distinguishing them, so the metes and bounds of the claims cannot be ascertained.

AND I MAKE this solemn declaration by virtue of the Statutory Declarations Act 1959, and subject to the penalties provided by that Act for the making of false statements in statutory

declarations, conscientiously believing the statements contained in this declaration to be true in every particular.

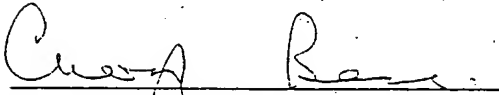
DECLARED at Melbourne, Victoria

This 12<sup>th</sup> day of November, 2001



Peter Adrian Walton Rogers

Before me:



(Signature of Witness)

Medical Practitioner

AUSTRALIA

*Patents Act 1990*

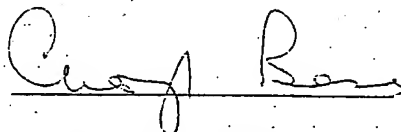
IN THE MATTER OF Australian Patent  
Application Serial No. 696764 by Human  
Genome Sciences, Inc.

-and-

IN THE MATTER OF Opposition thereto by  
Ludwig Institute for Cancer Research

THIS IS Exhibit PAWR-1  
referred to in the Statutory Declaration  
of Peter Adrian Walton Rogers  
made before me.

DATED this 12<sup>th</sup> Day of November, 2001

A handwritten signature in cursive script, appearing to read 'Craig Ross', written over a horizontal line.

(Signature of Witness)

Medical Practitioner

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(54) Title: HUMAN VASCULAR ENDOTHELIAL GROWTH FACTOR 2

(57) Abstract

Disclosed is a human VEGF2 polypeptide and DNA (RNA) encoding such VEGF2 polypeptides. Also provided is a procedure for producing such polypeptide by recombinant techniques and antibodies and antagonist against such polypeptide. Also disclosed is a method of using such polypeptide for stimulating wound healing and for vascular tissue repair. Also provided are methods of using the antagonists to inhibit tumor growth, inflammation and to treat diabetic retinopathy, rheumatoid arthritis and psoriasis. Diagnostic methods for detecting mutations in the VEGF2 coding sequence and alterations in the concentration of VEGF2 protein in a sample derived from a host are also disclosed.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. An isolated polynucleotide comprising a member selected from the group consisting of:
  - a) a polynucleotide encoding the full length polypeptide as set forth in SEQ ID NO: 2;
  - b) a polynucleotide encoding the mature protein portion of SEQ ID NO: 2;
  - c) a polynucleotide encoding the proprotein portion of SEQ ID NO: 2;
  - d) a polynucleotide encoding the human VEGF-2 polypeptide encoded by the cDNA in ATCC Deposit No. 97149;
  - e) a polynucleotide comprising the nucleotide sequence encoding a polypeptide comprising amino acid residues -46 to 373 of SEQ ID NO: 2;
  - f) a polynucleotide comprising the nucleotide sequence encoding a polypeptide comprising amino acid residues -23 to 373 of SEQ ID NO: 2;
  - g) a polynucleotide comprising the nucleotide sequence encoding a polypeptide comprising amino acids 1 to 373 of SEQ ID NO: 2;
  - h) a polynucleotide comprising the nucleotide sequence encoding a portion of the mature VEGF-2 polypeptide comprising amino acids 24 to 373 of SEQ ID NO: 2;
  - i) a polynucleotide comprising the nucleotide sequence encoding a polypeptide comprising amino acids -46 to 24 of SEQ ID NO: 2;
  - j) a polynucleotide comprising the nucleotide sequence encoding a polypeptide comprising amino acids -23 to 24 of SEQ ID NO: 2;
  - k) a polynucleotide comprising the nucleotide sequence encoding a polypeptide comprising amino acids 1 to 24 of SEQ ID NO: 2;
  - l) a polynucleotide fragment of the polynucleotide according to any one of a) to d) with the proviso that said polynucleotide fragment comprises at least 30 contiguous nucleotides of the polynucleotide of i), j) or k);
  - m) a polynucleotide fragment which hybridises to at least 30 contiguous nucleotides of the polynucleotide encoding amino acids -46 to 24 of SEQ ID NO: 2 under the following conditions: hybridisation in 0.5 M sodium peroxide



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NaPO<sub>4</sub>, 7% sodium dodecyl sulfate (SDS) at 65°C and washing with 0.5 x SSC, 0.1% SDS at 60°C or equivalent hybridisation stringency;

n) a polynucleotide comprising the complementary form of the polynucleotide according to any one of a) to m).

2. An isolated polynucleotide according to claim 1 wherein the polynucleotide encodes the full length polypeptide as set forth in SEQ ID NO: 2.
3. An isolated polynucleotide according to claim 1 wherein the polynucleotide encodes the mature protein portion of SEQ ID NO:2.
4. An isolated polynucleotide according to claim 1 wherein the polynucleotide encodes the proprotein portion of SEQ ID NO: 2.
5. An isolated polynucleotide according to claim 1 wherein the polynucleotide encodes the human VEGF-2 polypeptide encoded by the cDNA in ATCC Deposit No. 97149.
6. An isolated polynucleotide according to claim 1 wherein the polynucleotide encodes a polypeptide comprising amino acids -46 to 373 of SEQ ID NO: 2.
7. An isolated polynucleotide according to claim 1 wherein the polynucleotide encodes a polypeptide comprising amino acids -23 to 373 of SEQ ID NO: 2.
8. An isolated polynucleotide according to claim 1 wherein the polynucleotide encodes a polypeptide comprising amino acids 1 to 373 of SEQ ID NO: 2.
9. An isolated polynucleotide according to claim 1 wherein the polynucleotide encodes a portion of the mature VEGF-2 polypeptide comprising amino acids 24 to 373 of SEQ ID NO: 2.
10. An isolated polynucleotide according to claim 1 wherein the polynucleotide encodes





11. An isolated polynucleotide according to claim 1 wherein the polynucleotide encodes a polypeptide comprising amino acids -23 to 24 of SEQ ID NO: 2.
12. An isolated polynucleotide according to claim 1 wherein the polynucleotide encodes a polypeptide comprising amino acids 1 to 24 of SEQ ID NO: 2.
13. An isolated polynucleotide according to claim 1 wherein the polynucleotide comprises a fragment of the polynucleotide according to any one of a) to d) with the proviso that said polynucleotide fragment comprises at least 30 contiguous nucleotides of the polynucleotides of i), j) or k).
14. An isolated polynucleotide according to claim 1 wherein the polynucleotide comprises a fragment which hybridises to at least 30 contiguous nucleotides of the polynucleotide encoding amino acids -46 to 24 of SEQ ID NO: 2 under the following conditions: hybridisation in 0.5 M sodium phosphate NaPO<sub>4</sub>, 7% sodium dodecyl sulfate (SDS) at 65°C and washing with 0.5 x SSC, 0.1% SDS at 60°C or equivalent hybridisation stringency.
15. An isolated polynucleotide according to claim 1 wherein the polynucleotide comprises the complementary form of the polynucleotide according to any one of claims 2 to claim 14.
16. An isolated polypeptide comprising at least 30 amino acid residues and having VEGF2 biological activity further comprising a member selected from the group consisting of:
  - a) a polypeptide comprising the amino acid sequence of the full length polypeptide of SEQ ID NO:2;
  - b) a polypeptide comprising the amino acid sequence of the mature protein of SEQ ID NO: 2;
  - c) a polypeptide comprising the amino acid sequence of the proprotein portion of



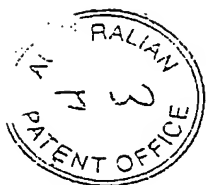
SEQ ID NO: 2;

- d) a polypeptide comprising the amino acid sequence of the mature polypeptide encoded by the cDNA contained in ATCC Deposit No. 97149;
- e) a polypeptide comprising amino acids -46 to 373 of SEQ ID NO: 2;
- f) a polypeptide comprising amino acids -23 to 373 of SEQ ID NO: 2;
- g) a polypeptide comprising amino acids 1 to 373 of SEQ ID NO: 2;
- h) a portion of the mature VEGF-2 polypeptide comprising amino acids 24 to 373 of SEQ ID NO: 2;
- i) a polypeptide comprising amino acids -46 to 24 of SEQ ID NO: 2;
- j) a polypeptide comprising amino acids -23 to 24 of SEQ ID NO: 2;
- k) a polypeptide comprising amino acids 1 to 24 of SEQ ID NO: 2;
- l) a polypeptide comprising an active fragment of the VEGF2 polypeptides according to any one of a) to d) with the proviso that part of said polypeptide fragment is encoded by at least 30 contiguous nucleotides of the polynucleotide encoding the polypeptide of any one of i), j) or k);
- m) a polypeptide fragment comprising an amino acid sequence encoded by a polynucleotide sequence which hybridises to at least 30 contiguous nucleotides of the polynucleotide encoding any one of the polypeptides of i), j) or k) under the following conditions: hybridisation in 0.5 M sodium peroxide  $\text{NaPO}_4$ , 7% sodium dodecyl sulfate (SDS) at 65°C and washing with 0.5 x SSC, 0.1% SDS at 60°C or equivalent hybridisation stringency.

- 17. An isolated polypeptide according to Claim 15 comprising the amino acid sequence of the full length polypeptide of SEQ ID NO: 2.
- 18. An isolated polypeptide according to Claim 16 comprising the amino acid sequence of the mature portion of SEQ ID NO: 2.
- 19. An isolated polypeptide according to Claim 16 comprising a polypeptide comprising the amino acid sequence of the proprotein portion of SEQ ID NO: 2.



20. An isolated polypeptide according to Claim 16 comprising the amino acid sequence of the mature VEGF-2 polypeptide encoded by the cDNA contained in ATCC Deposit No. 97149.
21. An isolated polypeptide according to Claim 16 comprising amino acids 46 to 373 of SEQ ID NO: 2.
22. An isolated polypeptide according Claim 16 comprising amino acids 23 to 373 of SEQ ID NO: 2.
23. An isolated polypeptide according to Claim 16 comprising amino acids 1 to 373 of SEQ ID NO: 2.
24. An isolated portion of the mature VEGF-2 polypeptide according to Claim 16 comprising amino acids 24 to 373 of SEQ ID NO: 2.
25. An isolated polypeptide according to Claim 16 comprising amino acids 46 to 24 of SEQ ID NO: 2.
26. An isolated polypeptide according to Claim 16 comprising amino acids 23 to 24 of SEQ ID NO: 2.
27. An isolated polypeptide according to Claim 16 comprising amino acids 1 to 24 of SEQ ID NO: 2.
28. An isolated polypeptide according to Claim 16 comprising a fragment of any one of the polypeptides of Claims 16 to 20 with the proviso that part of said polypeptide fragment is encoded by at least 30 contiguous nucleotides of the polynucleotide encoding the polypeptide of any one of Claims 25 to 27.
29. An isolated polypeptide according to Claim 16 comprising an amino acid sequence



encoded by a polynucleotide sequence which hybridises to at least 30 contiguous nucleotides of the polynucleotide encoding any one of the polypeptides of Claims 25 to 27 under the following conditions: hybridisation in 0.5 M sodium peroxide  $\text{NaPO}_4$ , 7% sodium dodecyl sulfate (SDS) at 65°C and washing with 0.5 x SSC, 0.1% SDS at 60°C or equivalent hybridisation stringency.

30. An isolated polypeptide according to any one of Claims 16 to 29 further comprising a heterologous polypeptide.
31. An isolated polypeptide according to any one of Claims 16 to 30 further comprising a homodimer.
32. An isolated polypeptide according to any one of Claims 16 to 31 wherein the polypeptide is glycosylated.
33. A composition comprising the polypeptide according to any one of Claims 16 to 32 or 40 and one or more pharmaceutically acceptable carriers and/or diluents.
34. A vector comprising the polynucleotide according to any one of Claims 1 to 15.
35. A recombinant vector comprising the polynucleotide according to any one of Claims 1-15 operatively associated with a regulatory sequence that controls gene expression.
36. A host cell comprising the polynucleotide according to any one of Claims 1-15 operably associated with a heterologous regulatory sequence or a vector comprising same.
37. The polynucleotide sequence according to any one of Claims 1-15 further comprising a heterologous polynucleotide.
38. The polynucleotide sequence of Claim 37 further comprising a polynucleotide which



encodes a heterologous polypeptide.

39. A method for producing a VEGF-2 polypeptide at least comprising the step of culturing the genetically engineered host cell of Claim 36 for a time and under conditions suitable for the expression of the polypeptide encoded by said polynucleotide to occur.
40. A polypeptide produced by the method of Claim 39.
41. A composition comprising the polynucleotide according to any one of Claims 1-15, 37, or 38 and one or more pharmaceutically acceptable carriers and/or diluents.
42. Use of the polynucleotide according to any one of Claims 1 to 15, 37 or 38 or the polypeptide according to any one of Claims 16 to 32 or 40 in the preparation of a medicament for the treatment of a patient having need of human VEGF-2 polypeptide.
43. An antibody which is capable of binding to the polypeptide according to any one of Claims 16 to 29 with the proviso that the antibody is not capable of binding to a polypeptide consisting of amino acid residues 24 to 373 of SEQ ID NO: 2 or a fragment thereof.
44. An antisense construct capable of binding to the polynucleotide according to any one of Claims 1-15 or a complementary form thereof with the proviso that the antisense construct is not capable of binding to a polynucleotide sequence encoding amino acids 24 to 373 of SEQ ID NO: 2 or a fragment thereof.
45. A method of stimulating proliferation of endothelial cells in a patient comprising administering to the patient the polypeptide according to any one of Claims 16 to 32 or 40 for a time and under conditions sufficient for the proliferation of endothelial cells to occur.



46. The method of Claim 45 wherein the patient has vasculature tissue damage.
47. The method of Claim 45 wherein the patient has a wound, tissue damage or bone damage.
48. The method of Claim 45 wherein the patient has ischemia.
49. The method of Claim 45 wherein the patient has myocardial infarction.
50. The method of Claim 45 wherein the patient has coronary artery disease, peripheral vascular disease or CNS vascular disease.
51. The method according to any one of Claims 46 to 50 wherein the stimulation of endothelial cell proliferation is capable of further stimulating angiogenesis.
52. The isolated polynucleotide according to any one of Claims 1 to 15, 37, 38 or 44 substantially as hereinbefore described with reference to the Figures and /or Examples.
53. The isolated polypeptide according to anyone of Claims 16 to 32 or 40 substantially as hereinbefore described with reference to the Figures and/or Examples.
54. The vector of Claim 34 substantially as hereinbefore described with reference to the Figures and/or Examples.
55. The host cell of Claim 36 substantially as hereinbefore described with reference to the Figures and/or Examples.
56. The method according to any one of Claims 39, 45 to 51 substantially as hereinbefore described with reference to the Figures and/or Examples.
57. The use according to Claim 42 substantially as hereinbefore described with reference



to the Figures and/or Examples.

58. The composition of Claim 33 or 41 substantially as hereinbefore described with reference to the Figures and/or Examples.

DATED this EIGHTH day of NOVEMBER, 1999

Human Genome Sciences, Inc.

DAVIES COLLISON CAVE

Patent Attorneys for the Applicant

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*Patents Act 1990*

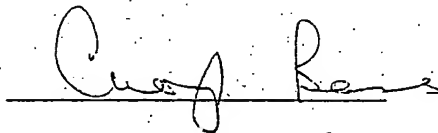
IN THE MATTER OF Australian Patent  
Application Serial No. 696764 by Human  
Genome Sciences, Inc.

-and-

IN THE MATTER OF Opposition thereto by  
Ludwig Institute for Cancer Research

THIS IS Exhibit PAWR-2  
referred to in the Statutory Declaration  
of Peter Adrian Walton Rogers  
made before me

DATED this 12<sup>th</sup> Day of November, 2001

A handwritten signature in cursive script, appearing to read 'Craig R...', written over a horizontal line.

(Signature of Witness)

Michael Proctor



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(54) Title: HUMAN TUMOR NECROSIS FACTOR DELTA AND EPSILON

(57) Abstract

The invention relates to human TNF delta and TNF epsilon polypeptides, polynucleotides encoding the polypeptides, methods for producing the polypeptides, in particular by expressing the polynucleotides, and agonists and antagonists of the polypeptides. The invention further relates to methods for utilizing such polynucleotides, polypeptides, agonists and antagonists for applications, which relate, in part, to research, diagnostic and clinical arts.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS

1. An isolated polynucleotide comprising a polynucleotide sequence selected from the group consisting of:-
  - (a) a polynucleotide sequence encoding a polypeptide comprising an amino acid sequence as set forth in SEQ ID NO:2;
  - (b) a polynucleotide sequence encoding a polypeptide comprising an amino acid sequence as set forth in SEQ ID NO:4;
  - (c) a polynucleotide sequence encoding a polypeptide comprising amino acid 39 to amino acid 233 of SEQ ID NO:2;
  - (d) a polynucleotide sequence having at least 70% identity to the polynucleotide sequence of (a), (b) or (c) and encoding a polypeptide having TNF-delta and/or TNF-epsilon activity;
  - (e) a polynucleotide sequence comprising at least 30 contiguous bases of the polynucleotide sequence of (a), (b), (c) or (d) and encoding a polypeptide having TNF-delta and/or TNF-epsilon activity;
  - (f) a polynucleotide sequence comprising at least 50 contiguous bases of the polynucleotide sequence of (a), (b), (c) or (d) and encoding a polypeptide having TNF-delta and/or TNF-epsilon activity;
  - (g) a polynucleotide sequence comprising a fragment of the polynucleotide sequence of (a), (b), (c) or (d), wherein said fragment encodes a polypeptide that retains TNF-delta and/or TNF-epsilon activity;
  - (h) a polynucleotide sequence of (g) which encodes at least 30-50 amino acids of SEQ ID No's 2 or 4; and
  - (i) a polynucleotide sequence which is complementary to the polynucleotide sequence of (a), (b), (c), (d), (e), (f), (g) or (h).
2. The polynucleotide of Claim 1 wherein the polynucleotide is DNA.
3. The polynucleotide of Claim 1 wherein the polynucleotide is RNA.
4. The polynucleotide of Claim 1 wherein the polynucleotide is genomic DNA.
5. The polynucleotide of Claim 2 which encodes a polypeptide comprising the amino acids of SEQ ID NO:2.



6. The polynucleotide of Claim 2 which encodes a polypeptide comprising amino acid 39 to 233 of SEQ ID NO:2.
7. The polynucleotide of Claim 2 which encodes a polypeptide comprising the amino acids of SEQ ID NO:4.
8. The polynucleotide of Claim 2 which encodes a polypeptide comprising the amino acids 1 to 188 of SEQ ID NO:4.
9. An isolated polynucleotide comprising a polynucleotide selected from the group consisting of:-
  - (a) a polynucleotide which encodes a mature polypeptide having the amino acid sequence expressed by the human cDNA contained in ATCC Deposit No. 97377;
  - (b) a polynucleotide which encodes a mature polypeptide having the amino acid sequence expressed by the human cDNA contained in ATCC Deposit No. 97457;
  - (c) a polynucleotide sequence having at least 70% identity to the polynucleotide sequence of (a) or (b) and encoding a polypeptide having TNF-delta and/or TNF-epsilon activity;
  - (d) a polynucleotide sequence comprising at least 30 contiguous bases of the polynucleotide of (a), (b) or (c) and encoding a polypeptide having TNF-delta and/or TNF-epsilon activity;
  - (e) a polynucleotide sequence comprising at least 50 contiguous bases of the polynucleotide sequence of (a), (b) or (c) and encoding a polypeptide having TNF-delta and/or TNF-epsilon activity;
  - (f) a polynucleotide comprising a fragment of the polynucleotide of (a), (b) or (c), wherein said fragment encodes a polypeptide that retains TNF-delta and/or TNF-epsilon activity;
  - (g) a polynucleotide sequence of (f) which encodes at least 30-50 amino acids of the polypeptide encoded by ATCC Deposit No's 97377 or 97457; and
  - (h) a polynucleotide which is complementary to the polynucleotide of (a), (b), (c), (d), (e), (f) or (g).

10. The polynucleotide of Claim 1 comprising from nucleotide 447 to nucleotide 1717 of SEQ ID NO:1.
11. The polynucleotide of Claim 1 comprising from nucleotide 332 to nucleotide 1717 of SEQ ID NO:1.
12. The polynucleotide of Claim 1 comprising from nucleotide 1 to nucleotide 564 of SEQ ID NO:3.
13. A vector comprising the DNA of Claim 2.
14. A host cell comprising the vector of Claim 13.
15. A process for producing a polypeptide comprising expressing from the host cell of Claim 14 the polypeptide encoded by the DNA in said vector.
16. A process for producing a cell comprising genetically engineering the cell with the vector of Claim 12 to thereby express the polypeptide encoded by the human cDNA contained in the vector.
17. A polypeptide comprising a member selected from the group consisting of:-
  - (a) a polypeptide having an amino acid sequence as set forth in SEQ ID NO:2;
  - (b) a polypeptide having an amino acid sequence as set forth in SEQ ID NO:4;
  - (c) a polypeptide comprising amino acid residues 39 to 233 of SEQ ID NO:2;
  - (d) a polypeptide comprising amino acid residues 1 to 188 of SEQ ID NO:4;
  - (e) a polypeptide having at least a 70% identity to the polypeptide of (a), (b), (c) or (d) and having TNF-delta and/or TNF-epsilon activity;
  - (f) a polypeptide comprising at least 30 contiguous amino acid residues of the polypeptide of (a), (b), (c), (d) or (e) and having TNF-delta and/or TNF-epsilon activity.

18. The polypeptide of Claim 17 wherein the polypeptide comprises amino acid 1 to amino acid 233 of SEQ ID NO:2.
19. The polypeptide of Claim 17 wherein the polypeptide comprises amino acid 39 to amino acid 233 of SEQ ID NO:2.
20. The polypeptide of Claim 17 wherein the polypeptide comprises amino acid 1 to amino acid 188 of SEQ ID NO:4.
21. A compound which inhibits activation of the polypeptide of Claim 17.
22. A method for the treatment of a patient having need of TNF delta comprising administering to the patient a therapeutically effective amount of the polypeptide of Claim 17.
23. A method for the treatment of a patient having need of TNF epsilon comprising administering to the patient a therapeutically effective amount of the polypeptide of Claim 17.
24. A method of Claim 22 wherein said therapeutically effective amount of the polypeptide is administered by providing to the patient DNA encoding said polypeptide and expressing said polypeptide *in vivo*.
25. A method of Claim 23 wherein said therapeutically effective amount of the polypeptide is administered by providing to the patient DNA encoding said polypeptide and expressing said polypeptide *in vivo*.
26. A method for the treatment of a patient having need to inhibit a TNF delta polypeptide comprising administering to the patient a therapeutically effective amount of the compound of Claim 21.
27. A method for the treatment of a patient having need to inhibit a TNF epsilon polypeptide comprising administering to the patient a therapeutically effective amount of the compound of Claim 21.

28. A process for diagnosing a disease or a susceptibility to a disease related to an under-expression of the polypeptide of Claim 17 comprising determining a mutation in a nucleic acid sequence encoding said polypeptide.
29. A diagnosis process comprising analysing for the presence of the polypeptide of claim 17 in a sample derived from a host.
30. A method for identifying compounds which bind to and inhibit activation of the polypeptide of Claim 17 comprising:-
  - contacting a cell expressing on the surface thereof a receptor for the polypeptide, said receptor being associated with a second component capable of providing a detectable signal in response to the binding of a compound to said receptor, with an analytically detectable TNF delta polypeptide and a compound under conditions to permit binding to the receptor; and
  - determining whether the compound binds to and inhibits the receptor by detecting the absence of a signal generated from the interaction of the TNF delta with the receptor.
31. An isolated polynucleotide comprising a polynucleotide sequence having at least 90% identity to a member of the group (a), (b), (c), (d), (e), (f), (g) or (h) in claim 1 and encoding a polypeptide having TNF-delta and/or TNF-epsilon activity.
32. An isolated polynucleotide comprising a polynucleotide sequence having at least 95% identity to a member of the group (a), (b), (c), (d), (e), (f), (g) or (h) in claim 1 and encoding a polypeptide having TNF-delta and/or TNF-epsilon activity.
33. An isolated polynucleotide comprising a polynucleotide sequence encoding a polypeptide with TNF-delta and/or TNF-epsilon activity and which hybridises to the complement of the polynucleotide set forth in SEQ ID NO:1 wherein said hybridisation occurs under conditions comprising hybridisation in a buffer consisting of 7% SDS, 0.5 M NaPO<sub>4</sub> pH 7.4 at 65°C and wash in a solution consisting of 0.5 x SSC, 0.1% SDS at 60°C.
34. An isolated polynucleotide comprising a polynucleotide sequence encoding a polypeptide with TNF-delta and/or TNF-epsilon activity and which hybridises to

the complement of the polynucleotide set forth in SEQ ID NO:3 wherein said hybridisation occurs under conditions comprising hybridisation in a buffer consisting of 7% SDS, 0.5 M NaPO<sub>4</sub> pH 7.4 at 65°C and wash in a solution consisting of 0.5 x SSC, 0.1% SDS at 60°C.

35. The isolated polypeptide of Claim 17 wherein said polypeptide comprises an amino acid sequence having at least 90% identity to a member of said group (a), (b), (c), (d), (e) or (f) and retains TNF-delta and/or TNF-epsilon activity.
36. The isolated polypeptide of Claim 17 wherein said polypeptide comprises an amino acid sequence having at least 95% identity to a member of said group (a), (b), (c), (d), (e) or (f) and retains TNF-delta and/or TNF-epsilon activity.
37. An isolated polynucleotide according to any one of Claims 1 to 12 or a vector according to Claim 13 or a host cell according to Claim 14 or a process according to Claims 15 or 16 or a polypeptide according to any one of Claims 17 to 20 or a compound according to Claim 21 or a method according to any one of Claims 22 to 30 or a polynucleotide according to any one of Claims 31 to 34 or a polypeptide according to Claim 35 or 36 substantially as hereinbefore described with reference to the Figures and/or Examples.



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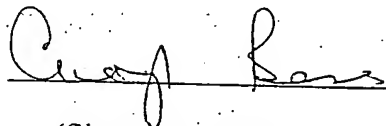
IN THE MATTER OF Australian Patent  
Application Serial No. 696764 by Human  
Genome Sciences, Inc.

-and-

IN THE MATTER OF Opposition thereto by  
Ludwig Institute for Cancer Research

THIS IS Exhibit PAWR-3  
referred to in the Statutory Declaration  
of Peter Adrian Walton Rogers  
made before me

DATED this 12<sup>th</sup> Day of November, 2001



(Signature of Witness)

Medical Practitioner

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<p>(21) International Application Number: PCT/US94/12880 (22) International Filing Date: 7 November 1994 (07.11.94) (71) Applicant (for all designated States except US): HUMAN GENOME SCIENCES, INC. [US/US]; 9410 Key West Avenue, Rockville, MD 20850-3338 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): YU, Guo-Liang [CN/US]; 13524 Straw Bale Lane, Darnestown, MD 20878 (US). NI, Jian [CN/US]; Apartment 204, 305 Westside Drive, Gaithersburg, MD 20878 (US). ROSEN, Craig, A. [US/US]; 22400 Rolling Hill Road, Laytonsville, MD 20882 (US). (74) Agents: OLSTEIN, Elliot, M. et al.; Carella, Byrne, Bain, Gilfillan, Cecchi, Stewart &amp; Olstein, 6 Becker Farm Road, Roseland, NJ 07068 (US).</p>		<p>(31) Designated States: AU, CA, CN, JP, KR, NZ, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  Published With international search report.</p>
<p>(54) Title: TUMOR NECROSIS FACTOR-GAMMA (57) Abstract  A human TNF-gamma polypeptide and DNA (RNA) encoding such polypeptide and a procedure for producing such polypeptide by recombinant techniques is disclosed. Also disclosed are methods for utilizing such polypeptide to inhibit cellular growth, for example in a tumor or cancer, for facilitating wound-healing, to provide resistance against infection, induce inflammatory activities, and stimulating the growth of certain cell types to treat diseases, for example restenosis. Also disclosed are diagnostic methods for detecting a mutation in the TNF-gamma nucleic acid sequence or an overexpression of the TNF-gamma polypeptide. Antagonists against such polypeptides and their use as a therapeutic to treat cachexia, septic shock, cerebral malaria, inflammation, arthritis and graft-rejection are also disclosed.</p>		

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. An isolated polynucleotide which encodes a tumor necrosis factor (TNF- $\gamma$ ) polypeptide, wherein said polynucleotide comprises a nucleotide sequence selected from the group consisting of:
  - (a) a nucleotide sequence encoding amino acid residues -25 to 149 of SEQ ID NO:2;
  - (b) a nucleotide sequence encoding the full-length polypeptide encoded by the human cDNA contained in ATCC Deposit No. 75927;
  - (c) a nucleotide sequence encoding amino acid residues 1 to 149 of SEQ ID NO:2;
  - (d) a nucleotide sequence encoding the mature polypeptide encoded by the human cDNA contained in ATCC Deposit No. 75927;
  - (e) a homologue or analogue of any one of (a) to (d), wherein said homologue or analogue is at least 70% identical to any one of (a) to (d) and encodes a peptide or polypeptide having TNF- $\gamma$  activity;
  - (f) a fragment of any one of (a) to (d), wherein said fragment comprises at least 30 contiguous nucleotides in length derived from any one of (a) to (d);
  - (h) a nucleotide sequence of at least 30 nucleotides in length that is capable of hybridizing to any one of (a) to (e) wherein said nucleotide sequence encodes TNF- $\gamma$  or is derived from a nucleotide sequence that encodes TNF- $\gamma$ ; and
  - (i) a nucleotide sequence complementary to any one of (a) to (h).
2. The isolated polynucleotide of claim 1 wherein said nucleotide sequence encodes amino acid residues -25 to 149 of SEQ ID NO:2.
3. The isolated polynucleotide of claim 1 wherein said nucleotide sequence encodes amino acid residues 1 to 149 of SEQ ID NO:2;
4. The isolated polynucleotide of claim 1 wherein said nucleotide sequence encodes the full-length polypeptide encoded by the human cDNA contained in ATCC Deposit



No. 75927.

5. The isolated polynucleotide of claim 1 wherein said nucleotide sequence encodes the mature polypeptide encoded by the human cDNA contained in ATCC Deposit No. 75927.

6. The isolated polynucleotide of claim 1 wherein the percentage identity to a homologue or analogue of any one of (a) to (d) is at least 95%.

7. The isolated polynucleotide of claim 1 wherein the fragment of any one of (a) to (d) comprises at least 50 contiguous nucleotides in length derived from any one of (a) to (d).

8. An isolated polynucleotide which encodes a tumor necrosis factor (TNF- $\gamma$ ) polypeptide, wherein said polynucleotide comprises the nucleotide sequence set forth as SEQ ID NO:1 or at least 30 contiguous nucleotide residues derived therefrom.

9. An isolated polynucleotide which encodes a tumor necrosis factor (TNF- $\gamma$ ) polypeptide, wherein said polynucleotide comprises a nucleotide sequence which is identical to the nucleotide sequence of the human cDNA contained in ATCC Deposit No. 75927 or at least 30 contiguous nucleotide residues derived therefrom.

10. An isolated polynucleotide that comprises a nucleotide sequence that is complementary to the nucleotide sequence of the isolated polynucleotide according to claim 8.

11. An isolated polynucleotide that comprises a nucleotide sequence that is complementary to the nucleotide sequence of the isolated polynucleotide according to claim 9.



12. The isolated polynucleotide according to any one of claims 1 to 11 comprising DNA.
13. The isolated polynucleotide of claim 12 wherein the DNA is genomic DNA.
14. A vector comprising the isolated polynucleotide according to any one of claims 1 to 13.
15. A host cell transformed or transfected with the polynucleotide according to any one of claims 1 to 13 or the vector of claim 14.
16. The isolated polynucleotide according to any one of claims 1 to 13 in operable connection with a heterologous regulatory sequence which controls gene expression.
17. A method of producing a polynucleotide which encodes TNF- $\gamma$  comprising hybridizing at least 30 contiguous nucleotides derived from SEQ ID NO:1 under stringent hybridization conditions with nucleic acid for a time and under conditions sufficient for hybridization to occur and then detecting said hybridization.
18. A method of producing a tumor necrosis factor (TNF- $\gamma$ ) polypeptide, said method comprising incubating or growing the host cell of claim 15 for a time and under conditions sufficient for expression of the polypeptide encoded by the introduced polynucleotide in said cell to occur.
19. A method of producing a cell capable of expressing a a tumor necrosis factor (TNF- $\gamma$ ) polypeptide, said method comprising transforming or transfecting a cell with the vector of claim 14.
20. A recombinant tumor necrosis factor (TNF- $\gamma$ ) polypeptide when produced by the method of claim 18.



21. An isolated or recombinant tumor necrosis factor (TNF- $\gamma$ ) polypeptide which comprises an amino acid sequence selected from the group consisting of:
  - (a) amino acid sequence shown as residues -25 to 149 of SEQ ID NO:2;
  - (b) amino acid residues 1 to 149 of SEQ ID NO:2;
  - (c) the amino acid sequence of the full-length polypeptide encoded by the human cDNA contained in ATCC Deposit No. 75927;
  - (d) the amino acid sequence of the mature polypeptide encoded by the human cDNA contained in ATCC Deposit No. 75927;
  - (e) the amino acid sequence of an analogue or derivative of any one of (a) to (d) wherein said analogue or derivative is at least 70% identical to any one of (a) to (d) and has tumor necrosis factor (TNF- $\gamma$ ) activity; and
  - (f) a fragment of any one of (a) to (d) that is encoded by at least 30 contiguous nucleotide residues present in SEQ ID NO:1 or the human cDNA contained in ATCC Deposit No. 75927 or a degenerate nucleotide sequence thereto.
22. The isolated or recombinant polypeptide of claim 21 comprising amino acid residues -25 to 149 of SEQ ID NO:2.
23. The isolated or recombinant polypeptide of claim 21 comprising amino acid residues 1 to 149 of SEQ ID NO:2.
24. The isolated or recombinant polypeptide of claim 21 comprising the amino acid sequence of the full-length polypeptide encoded by the human cDNA contained in ATCC Deposit No. 75927.
25. The isolated or recombinant polypeptide of claim 21 comprising the amino acid sequence of the mature polypeptide encoded by the human cDNA contained in ATCC Deposit No. 75927.
26. An antibody which binds specifically to the isolated or recombinant



polypeptide according to any one of claims 20 to 25.

27. A compound which antagonises the activity of the polypeptide according to any one of claims 20 to 25 or a naturally-occurring form of said polypeptide, wherein said compound was not known previously to antagonise the activity of said polypeptide.
28. A compound which agonises the activity of the polypeptide according to any one of claims 20 to 25 or a naturally-occurring form of said polypeptide, wherein said compound was not known previously to agonise the activity of said polypeptide.
29. A method of treatment of a patient having need of human TNF- $\gamma$  comprising administering to the patient a therapeutically effective amount of the polypeptide according to any one of claims 20 to 25 or the compound of claim 28 or a composition comprising said polypeptide or said compound.
30. A method of treatment of a patient having need to inhibit human TNF- $\gamma$  comprising administering to the patient a therapeutically effective amount of the compound according to claim 27 or a composition comprising said compound.
31. A pharmaceutical composition comprising the polypeptide according to any one of claims 20 to 25 in combination with a pharmaceutically acceptable carrier.
32. The method of claim 29 wherein the polypeptide according to any one of claims 20 to 25 or the composition comprising said polypeptide is administered by providing to the patient DNA encoding said polypeptide and expressing said polypeptide *in vivo*.
33. A method of identifying a modulator of human TNF- $\gamma$  activity comprising:
- (a) combining endothelial cells, Con A, [ $^3$ H]thymidine, and a compound to be tested for modulatory activity with the isolated or recombinant polypeptide according





to any one of claims 20 to 25 for a time and under conditions sufficient for human TNF- $\gamma$  activity to stimulate [ $^3$ H]thymidine incorporation into said endothelial cells; and

(b) determining the level of [ $^3$ H]thymidine incorporation in (a) compared to the [ $^3$ H]thymidine incorporation obtained in the absence of said compound, wherein a variation in [ $^3$ H]thymidine incorporation indicates that said compound is a modulator of TNF- $\gamma$  activity.

34. The method of claim 33 wherein the modulator of human TNF- $\gamma$  is an agonist of human TNF- $\gamma$  activity.

35. The method of claim 33 wherein the modulator of human TNF- $\gamma$  is an antagonist of human TNF- $\gamma$  activity.

36. A compound which agonises the activity of human TNF- $\gamma$  when identified by the method of claim 34, wherein said compound was not known previously to agonise TNF- $\gamma$  activity.

37. A compound which antagonises the activity of human TNF- $\gamma$  when identified by the method of claim 35, wherein said compound was not known previously to antagonise TNF- $\gamma$  activity.

38. A method of diagnosing a disease in a subject or a susceptibility of a subject to a disease, wherein said disease is related to a mutation in the TNF- $\gamma$ -encoding nucleotide sequences of said subject, and wherein said method comprises determining a mutation in a nucleotide sequence of said subject which encodes TNF- $\gamma$  using the isolated polynucleotide according to any one of claims 1 to 13 or a chemically-synthesised oligonucleotide comprising an identical nucleotide sequence thereto or a vector comprising said nucleotide sequence.



39. The method according to claim 38 wherein the mutation is determined by comparing the nucleotide sequence of the subject which encodes TNF- $\gamma$  with the nucleotide sequence of the nucleotide according to any one of claims 1 to 13, and wherein a difference between said nucleotide sequences is indicative of the mutation.
40. The method according to claim 38 or 39 when used to diagnose a tumor or a susceptibility to a tumor in a subject.
41. A method of diagnosing TNF- $\gamma$  expression in a subject comprising analysing a sample derived from said subject for the presence of the polypeptide according to any one of claims 20 to 25.
42. The method according to claim 41 comprising contacting a biological sample derived from said subject with an antibody molecule capable of binding to the isolated or recombinant polypeptide according to any one of claims 20 to 25 for a time and under conditions sufficient for an antigen-antibody complex to form and then detecting said complex formed.
43. A method of inhibiting tumor cell growth in a subject comprising administering to the subject a therapeutically effective amount of the polypeptide according to any one of claims 20 to 25 or the pharmaceutical composition according to claim 31 for a time and under conditions sufficient for tumor growth to be inhibited.
44. Use of the polypeptide according to any one of claims 20 to 25 in the manufacture of a medicament to inhibit tumor cell growth, induce cell adhesion or promote endothelial cell growth in a human or animal subject.
45. Use of the isolated polynucleotide according to any one of claims 1 to 13 or the vector of claim 14 in the manufacture of a medicament to inhibit tumor cell growth, induce cell adhesion or promote endothelial cell growth in a human or animal subject.



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46. The vector of claim 14 substantially as hereinbefore described with reference to the Figures and/or Examples.
47. The host cell of claim 15 substantially as hereinbefore described with reference to the Figures and/or Examples.
48. The method according to claim 18 substantially as hereinbefore described with reference to the Figures and/or Examples.
49. The method according to claim 19 substantially as hereinbefore described with reference to the Figures and/or Examples.

DATED this TWENTY FOURTH day of JUNE, 1999  
Human Genome Sciences, Inc.  
by DAVIES COLLISON CAVE  
Patent Attorneys for the Applicants



AUSTRALIA

*Patents Act 1990*

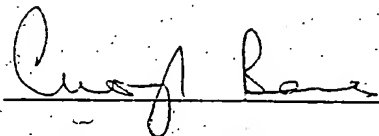
IN THE MATTER OF Australian Patent  
Application Serial No. 696764 by Human  
Genome Sciences, Inc.

-and-

IN THE MATTER OF Opposition thereto by  
Ludwig Institute for Cancer Research

THIS IS Exhibit PAWR-4  
referred to in the Statutory Declaration  
of Peter Adrian Walton Rogers  
made before me

DATED this 12<sup>th</sup> Day of November, 2001

A handwritten signature in cursive script, appearing to read "Craig Lane", written over a horizontal line.

(Signature of Witness)

Medical Practitioner

9.9.88/6 MB

# Antibodies

## A LABORATORY MANUAL

Ed Harlow

Cold Spring Harbor Laboratory

David Lane

Imperial Cancer Research Fund Laboratories



Cold Spring Harbor Laboratory  
1988

## Antibodies A LABORATORY MANUAL

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### *Designing the Peptide*

Probably the most frequently asked question concerning synthetic peptides is what sequence should be used for the immunogen (reviewed in Doolittle 1986). Although there is no one correct answer, enough anti-peptide antibodies have been raised to make suggestions for peptide choices. However, preparing anti-peptide antibodies is still an empirical exercise. What works well for one immunogen may fail completely for another.

### *Choosing the Appropriate Peptide Sequence*

With careful synthesis, coupling, and immunizations, most sequences can be used to induce antibodies specific for the peptide itself. When considering which sequence to use, most people actually want to know how likely will it be that the anti-peptide antibodies will recognize the native protein. Early work suggested that peptides containing hydrophilic amino acids (Hopp and Woods 1981, 1983; Kyte and Doolittle 1982) and proline residues were more likely to be exposed on the surface of the native protein than other sequences, and many peptides have been prepared using these criteria. In assessing the value of these criteria, hydrophilicity is required but is not sufficient to predict the surface location of a particular sequence. Many strongly hydrophilic amino acid sequences are buried in water pockets or form inter- or intramolecular bonds and are thus excluded from interactions with anti-native antibodies. Therefore, hydrophilicity can be thought of as required but not sufficient for choosing peptide sequences (see p. 661 for hydrophilicity values). Hydrophilic peptides are also more likely to be soluble for coupling reactions.

The presence of proline residues in synthetic peptides originally was suggested because  $\beta$ -turns often form portions of known epitopes. However, the presence of proline residues in peptides does not have much predictive value when antisera are tested for binding to the surface of native proteins. Although many excellent anti-peptide antisera have been prepared against sequences with proline residues, there is not sufficient evidence to target prolines when designing peptides.

More recently, several workers have noted that carboxy-terminal sequences often are exposed and can be targeted for anti-peptide sequences. Although using carboxy-terminal sequences does not guarantee that the resulting antibodies will recognize the native protein, a surprisingly high percentage will. Similarly, many amino-terminal regions are exposed, and these also may make good targets.

Another potentially useful parameter for selecting peptide sequences is the "mobility" of the amino acid residues. Originally, it was noted that the regions of a protein that become epitopes often have a higher temperature than other regions, as determined by NMR and X-ray structure (Moore and Williams 1980; Robinson et al. 1983; Tainer et al. 1984; Westhof et al. 1984). Higher temperature in crystallography and NMR distinguishes regions that are more mobile from

regions that are more static. These observations have led to the suggestion that stretches of amino acids that are more flexible are more likely to be epitopes. In the preparation of anti-peptide antibodies, when a peptide is coupled to a carrier molecule, it has a different local environment than in the original protein. When choosing a sequence for antibody production, a region of the protein that is more flexible will be more likely over time to form a structure that is similar to the peptide-carrier conjugate. Although the measure of mobility may become a useful criterion for selecting good peptide sequences, it has not been tested in enough detail to determine whether it will have any predictive value.

At present, a reasonable order of suggestions for choosing peptide sequences would be:

1. If possible, use more than one peptide.
2. Use the carboxyl-terminal sequence if it is hydrophilic and if a suitable coupling group is available or can be added.
3. Use the amino-terminal sequence if it is hydrophilic and if a suitable coupling group is available or can be added.
4. Use internal hydrophilic regions; perhaps using longer peptides.

### *Size of the Peptide*

The smallest synthetic peptides that will consistently elicit antibodies that bind to the original protein are 6 residues in length. Responses to smaller peptides are typically weak or will not recognize the protein of interest, either in a native or denatured state. Since epitopes consisting of smaller regions have been reported, the lower limit presumably reflects the difficulty of recognizing the smaller peptides coupled to carriers. With peptides of 6 amino acids or slightly larger, the responses vary. Some will generate good antibodies and some will not. Generally, peptides of approximately 10 residues should be used as a lower limit for coupling.

In the literature two strategies are suggested for peptide length. One school suggests using long peptides (up to 40 amino acids long) to increase the number of possible epitopes, while other authors argue that smaller peptides are adequate and their use ensures that the site-specific character of anti-peptide antibodies is retained. Both strategies have been used successfully. Two important preliminary questions to consider are: (1) Does the anti-peptide serum need to recognize the native protein? If so, use longer peptides or prepare anti-peptide antisera against multiple peptides. (2) How good is your peptide synthesis facility? Peptides over 20 residues in length are increasingly difficult to synthesize, yielding products with inappropriate side reactions. Longer peptides also are more likely to contain residues that make the coupling to carrier molecules more difficult. The correct decision between peptides with 10-15 residues and longer peptides will depend on the experimental design and will normally be a compromise between these factors. The safest choice, but also the most expensive, will be to prepare multiple small peptides of 10-15 amino acids in length from various regions of the sequence.



### *Coupling Strategy*

When choosing the sequence for a synthetic peptide, one factor that often is overlooked is the method of coupling. Most coupling methods rely on the presence of free amino, sulfhydryl, phenolic, or carboxylic acid groups. Free amino groups used for coupling will be found on lysine side chains or on the amino-terminal residue. Sulfhydryl groups are found on cysteine side chains, phenolic groups on tyrosines, and carboxylic acid groups on aspartic acids, glutamic acids, and the carboxy-terminal residue. Coupling methods should be used that link the peptide to the carrier via either the carboxy- or amino-terminal residue. When preparing antibodies against the carboxy-terminal region of the protein, the coupling should be done through the amino terminus of the peptide. Similarly, the coupling for amino terminal fragments should be done through the carboxy-terminal region of the peptide. For internal fragments, the major consideration is that the peptide be coupled by an end and not through a central residue.

The easiest strategy to manipulate the type of coupling is to add an extra amino acid on either the amino or carboxyl terminus to allow simple, one-site coupling to the carrier. Any coupling method that potentially can bind to an internal residue should be avoided. Similarly, coupling methods should be chosen that will bind to only one amino acid, if possible. If multiple coupling sites are possible, they should be localized to either the amino or carboxyl terminus, and the coupling should be adjusted to link only through one site per peptide on average. It is important to remember that it is often easier to use different peptides than design elaborate coupling schemes.

### *Choosing the Appropriate Carrier*

Many different carrier proteins can be used for coupling with synthetic peptides. The two most commonly used are keyhole limpet hemacyanin (KLH) and bovine serum albumin (BSA). Both work well in most cases, but each has disadvantages. Because of its large size, KLH is more likely to precipitate during cross-linking, and this can make handling KLH difficult in some cases. On the other hand, BSA is very soluble, but often is a good immunogen in its own right. For most purposes, either carrier will be adequate. Use whichever is more convenient.

Three other carriers that are used occasionally are ovalbumin, mouse serum albumin, or rabbit serum albumin. Ovalbumin can be used as a good carrier for most purposes. It is also a good choice for a second carrier when checking that antibodies are specific for the peptide itself and not the carrier. MSA or RSA may be used when the antibody response to the carrier molecule must be kept to a minimum.

BSA has 59 lysine (30-35 are available for coupling), 19 tyrosine, 35 cysteine, 39 aspartic acid, and 59 glutamic acid residues. Ovalbumin has 20 lysine, 10 tyrosine, 6 cysteine, 14 aspartic acid, and 33 glutamic acid residues.



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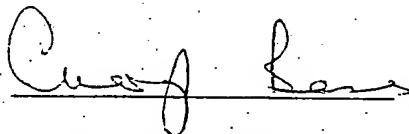
IN THE MATTER OF Australian Patent  
Application Serial No. 696764 by Human  
Genome Sciences, Inc.

-and-

IN THE MATTER OF Opposition thereto by  
Ludwig Institute for Cancer Research

THIS IS Exhibit PAWR-5  
referred to in the Statutory Declaration  
of Peter Adrian Walton Rogers  
made before me

DATED this 12<sup>th</sup> Day of November, 2001

A handwritten signature in cursive script, appearing to read "Craig Lane", written over a horizontal line.

(Signature of Witness)

medical practitioner

# From genes to protein structure and function: novel applications of computational approaches in the genomic era

Jeffrey Skolnick and Jacquelyn S. Fetrow

The genome-sequencing projects are providing a detailed 'parts list' of life. A key to comprehending this list is understanding the function of each gene and each protein at various levels. Sequence-based methods for function prediction are inadequate because of the multifunctional nature of proteins. However, just knowing the structure of the protein is also insufficient for prediction of multiple functional sites. Structural descriptors for protein functional sites are crucial for unlocking the secrets in both the sequence and structural-genomics projects.

**G**enome-sequencing projects are providing a detailed 'parts list' for life. Unfortunately, this list, a portion of which represents the amino acid sequence of all the proteins in a given genome, does not come with an instruction manual. That is, given the genome's sequences, one does not necessarily know straight away which regions encode proteins, which serve a regulatory role and which are responsible for the structure and replication of the DNA itself.

This is not unlike giving a child a list of parts necessary to create a working automobile. Without the necessary expertise, creating the final, working car from just the initial parts list is a nearly impossible task. Similarly, understanding how to create a complete, functioning cell given just the sequence of nucleotides found in an organism's genome is a complex problem.

## What is a protein function?

After a genome is sequenced and its complete parts list determined, the next goal is to understand the function(s) of each part, including that of the proteins. What do we mean by protein function, the focus of this article?

Function has many meanings. At one level, the protein could be a globular protein, such as an enzyme, hormone or antibody, or it could be a structural or membrane-bound protein. Another level is its biochemical function, such as the chemical reaction and the substrate specificity of an enzyme. The regulatory molecules or cofactors that bind to a protein are also levels of biochemical function.

At the cellular level, the protein's function would involve its interaction with other macromolecules and the function and cellular location of such complexes. There is also the protein's physiological function; that is, in which metabolic pathway the protein is involved or what physiological role it performs in the organism. Finally, the phenotypic function is the role played by the protein in the total organism, which is observed by deleting or mutating the gene encoding the protein.

Obviously, the complete characterization of protein function is difficult but efforts are under way at all levels<sup>1-4</sup>, including cellular function<sup>3,6</sup>. In this article, however, we focus on identifying the biochemical function of a protein given its sequence, a problem that is amenable to molecular approaches.

## Sequence-based approaches to function prediction

The sequence-to-function approach is the most commonly used function-prediction method. This robust field is well developed and, in the interest of space limitations, we will merely present a brief overview.

There are two main flavors of this approach: sequence alignment<sup>7-9</sup>; and sequence-modif methods such as Prosite<sup>10</sup>, Blocks<sup>11</sup>, Prints<sup>12,13</sup> and Emotif<sup>14</sup>. Both the alignment and the modif methods are powerful but a recent analysis has demonstrated their significant limitations<sup>15</sup>, suggesting that these methods will increasingly fail as the protein-sequence databases become more diverse.

An extension of these approaches that combines protein-sequence with structural information has been developed and some successes have been reported<sup>16</sup>. However, this method still applies the structural information in a one-dimensional, 'sequence-like' fashion and fails to take into account the powerful three-dimensional information displayed by protein structures.

In addition, proteins can gain and lose function during evolution and may, indeed, have multiple functions in the cell (Box 1). Sequence-to-function methods cannot specifically identify these complexities. Inaccurate use of sequence-to-function methods has led to significant function-annotation errors in the sequence databases<sup>17</sup>.

## An alternative approach

An alternative, complementary approach to protein-function prediction uses the sequence-to-structure-to-function paradigm. Here, the goal is to determine the structure of the protein of interest and then to identify the functionally important residues in that structure. Using the chemical structure itself to identify functional sites is more in line with how the protein actually works.

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In a sense, this is one long-term goal of 'structural genomics' projects<sup>18,19</sup>, which are designed to determine all possible protein folds experimentally, just as genome-sequencing projects are determining all protein sequences<sup>20</sup>. This is in contrast to traditional structural-biology approaches, in which one knows the protein's function first and only then, if the function is sufficiently important, determines its structure.

It is implicitly assumed that having the protein's structure will provide insights into its function, thereby furthering the goals of the human-genome-sequencing project. However, knowing a protein's three-dimensional structure is insufficient to determine its function (Box 2). What we really need to analyse and predict the multifunctional aspects of proteins is a method specifically to recognize active sites and binding regions in these protein structures.

#### Active-site identification

In order to use a structure-based approach to function prediction, one must identify the key residues responsible for a given biochemical activity. For many years, it has been suggested that the active sites in proteins are better conserved than the overall fold. Taken to the limit, this suggests that one could not only identify distant ancestors with the same global fold and the same activity but also proteins with similar functions but distantly related, or possibly unrelated, global folds.

The validity of this suggestion was demonstrated empirically by Nussinov and co-workers, who showed that the active sites of eukaryotic serine proteases, subtilisins and sulphhydryl proteases exhibit similar structural motifs<sup>21</sup>. Furthermore, in a recent modeling study of *Saccharomyces cerevisiae* proteins, protein functional sites were found to be more conserved than other parts of the protein models<sup>22</sup>. Similarly, it has been demonstrated that the catalytic triad of the  $\alpha/\beta$  hydrolases is structurally better conserved than other histidine-containing triads<sup>23</sup>. A comparison of the structure of the hydrolase catalytic triad to other histidine-containing triads shows a distinct bimodal distribution, while a similar analysis done with a randomly selected triad shows a unimodal distribution (Fig. 1).

Kasuya and Thornton<sup>24</sup> generalized this example by creating structural analogs of a few Prosite sequence motifs<sup>10</sup>. For the 20 most-frequently occurring Prosite patterns, the associated local structure is quite distinct. These results provide clear evidence that enzyme active sites are indeed more highly conserved than other parts of the protein.

#### Identifying active sites in experimental structures

Historically, several groups have attempted to identify functional sites in proteins; these efforts were directed at protein engineering or building functional sites in places where they did not previously exist. This has been successfully accomplished for several metal-binding sites<sup>25-33</sup>. However, highly accurate functional-site descriptors of the backbone and side-chain atoms were required, fueling the belief that significant atomic detail is required in site descriptors for function identification.

Highly detailed residue side-chain descriptors of the active sites of serine proteases and related proteins have been used to identify functional sites<sup>3</sup>. The use of these highly detailed motifs has led to the identification of

### Box 1. Proteins are multifunctional

A common protein characteristic that makes functional analysis based only on homology especially difficult is the tendency of proteins to be multifunctional. For instance, lactate dehydrogenase binds NAD, substrate and zinc, and performs a redox reaction. Each of these occurs at different functional sites that are in close proximity and the combination of all four sites creates the fully functional protein.

Other examples of multifunctional proteins are the nucleic-acid-binding proteins. For instance, DNA regulatory proteins often contain a DNA-binding domain, a multimerization domain and additional sites that bind regulatory proteins; a classic example is RecA<sup>59</sup>. The 3C rhinovirus protease exhibits a proteolytic function as well as an RNA-binding function<sup>60,61</sup>. Transcription factors are also complex, multifunctional proteins<sup>62</sup>. It is becoming increasingly important to recognize each of these different functions of gene products of a newly sequenced gene.

The serine-threonine-phosphatase superfamily is a prime example of the difficulties of using standard sequence analysis to recognize the multiple functions found in single proteins. This large protein family is divided into a number of subfamilies, all of which contain an essential phosphatase active site. Subfamilies 1, 2A and 2B exhibit 40% or more sequence identity between them<sup>63</sup>. However, each of these subfamilies is apparently regulated differently in the cell<sup>64-67</sup> and observation suggests that there are different functional sites at which regulation can occur. Because the sequence identity between subfamilies is so high, standard sequence-similarity methods could easily misclassify new sequences as members of the wrong subfamily if the functional sites are not carefully considered, as was recently demonstrated<sup>43</sup>.

These are but a few examples of the multifunctionality of proteins. The recognition of this multifunctional nature is of critical importance to the genomics field. Useful functional-annotation methods must consider all of the specific functions in a given protein and will not just provide a general classification of function.

several novel functional sites in known, high-quality protein structures<sup>3,34</sup>. More automated methods for finding spatial motifs in protein structures have also been described<sup>21,34-40</sup>.

Unfortunately, most of these methods require the exact placement of atoms within protein backbones and side chains, and so have not been shown to be relevant to inexact predicted structures. Recently, however, we described the production of fuzzy, inexact descriptors of protein functional sites<sup>15</sup>. As we wish to apply the descriptors to experimental structures as well as to predicted protein models, we used only carbon atoms and side-chain centers-of-mass positions. We call these descriptors 'fuzzy functional forms' (FFFs) and have created them for both the disulfide-oxidoreductase<sup>13,41</sup> and  $\alpha/\beta$ -hydrolase catalytic active sites<sup>23</sup>.

The disulfide-oxidoreductase FFF was applied to screen high-resolution structures from the Brookhaven protein database<sup>42</sup>. In a dataset of 364 protein structures, the FFF accurately identified all proteins known to exhibit the disulfide-oxidoreductase active site<sup>15</sup>. In a larger dataset of 1501 proteins, the FFF again accurately identified all proteins with the active site. In addition, it identified another protein, 1fjm, a serine-threonine phosphatase. This result was initially discouraging but subsequent sequence alignment and clustering analysis strongly suggested that this putative site might indeed be a site of redox regulation in the serine-threonine phosphatase-1 subfamily<sup>43</sup>. If confirmed by experiment, this result will highlight the advantages of using structural descriptors to analyse multiple functional sites in proteins. It will also highlight the fact that human

## Box 2. Knowing a protein's structure does not necessarily tell you its function

Because proteins can have similar folds but different functions<sup>58,69</sup>, determining the structure of a protein may or may not tell you something about its function. The most well-studied example is the ( $\alpha/\beta$ )<sub>3</sub> barrel enzymes, of which triose-phosphate isomerase (TIM) is the archetypal representative. Members of this family have similar overall structures but different functions, including different active sites, substrate specificities and cofactor requirements<sup>70,71</sup>.

Is this example common? Our own analysis of the 1997 SCOP database<sup>68</sup> shows that the five largest fold families are the ferredoxin-like, the ( $\alpha/\beta$ ) barrels, the knottins, the immunoglobulin-like and the flavodoxin-like fold families with 22, 18, 13, 9 and 9 subfamilies, respectively (Fig. 1). In fact, 57 of the SCOP fold families consist of multiple superfamilies. These data only show the tip of the iceberg, because each superfamily is further composed of protein families and each individual family can have radically different functions. For example, the ferredoxin-like superfamily contains families identified as Fe-S ferredoxins, ribosomal proteins, DNA-binding proteins and phosphatases, among others.

After this article was submitted, a much-more-detailed analysis of the SCOP database was published<sup>72</sup>. This finds a broad function-structure correlation for some structural classes, but also finds a number of ubiquitous functions and structures that occur across a number of families. The article provides a useful analysis of the confidence with which structure and function can be correlated<sup>72</sup>. Knowing the protein structure by itself is insufficient to annotate a number of functional classes and is also insufficient for annotating the specific details of protein function.

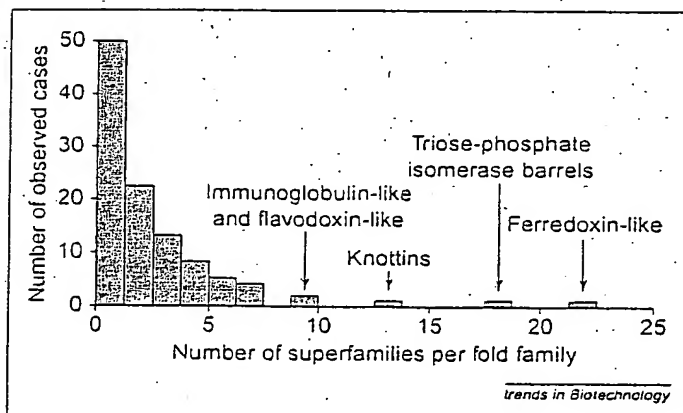


Figure 1

Histogram of the numbers of superfamilies found in each SCOP fold family. These data clearly show that proteins with similar structures can have different functions and demonstrate the difficulty of assigning protein function based simply on the three-dimensional structure. The data were taken from the 1997 distribution of SCOP (<http://scop.mrc-lmb.cam.ac.uk/scop>). For a more-detailed analysis, see Ref. 72.

observation alone is no longer adequate for identifying all functional sites in known protein structures.

To date, the use of structure to identify function has largely focused on high-resolution structures and highly detailed descriptors of protein functional sites. However, the creation of inexact descriptors for functional sites opens the way to the application of these methods to inexact, predicted protein models. The question remains: how good does a model have to be in order to use FFFs to identify its active sites?

## The state of the art in structure-prediction methods

For proteins whose sequence identity is above ~30%, one can use homology modeling to build the structure<sup>44</sup>. However, structure prediction is far more difficult for proteins that are not homologous to proteins with known structure. At present, there are two approaches for these sequences: *ab initio* folding<sup>45-48</sup> and threading<sup>49-53</sup>.

In *ab initio* folding, one starts from a random conformation and then attempts to assemble the native structure. As this method does not rely on a library of pre-existing folds, it can be used to predict novel folds. The recent CASP3 protein-structure-prediction experiment (<http://PredictionCenter.llnl.gov/CASP3>) involved the blind prediction of the structure of proteins whose actual structure was about to be experimentally determined. These results indicate that considerable progress has been made<sup>46,54</sup>. For helical and  $\alpha/\beta$  proteins with less than 110 residues, structures were often predicted whose backbone root-mean-square deviation (RMSD) from native ranged from 4–7 Å. Progress is being made with the  $\beta$  proteins, too, although they remain problematic. Because *ab initio* methods can identify novel folds, these methods could be used to help to select sequences likely to yield novel folds in experimental structural-genomics projects.

Another approach to tertiary-structure prediction is threading. Here, for the sequence of interest, one attempts to find the closest matching structure in a library of known folds<sup>52,55</sup>. Threading is applicable to proteins of up to 500 residues or so and is much faster than *ab initio* approaches. However, threading cannot be used to obtain novel folds.

## *Ab initio* predicted models can be used for automatic protein-function prediction

The results of the recent CASP3 competition suggest that current modeling methods can often (but not always) create inexact protein models. Are these structures useful for identifying functional sites in proteins? Using the *ab initio* structure-prediction program MONSTER, the tertiary structure of a glutaredoxin, 1ego, was predicted<sup>56</sup>. For the lowest-energy model, the overall backbone RMSD from the crystal structure was 5.7 Å.

To determine whether this inexact model could be used for function identification, the sets of correctly and incorrectly folded structures were screened with the FFF for disulfide-oxidoreductase activity<sup>15</sup>. The FFF uniquely identified the active site in the correctly folded structure but not in the incorrectly folded ones (Fig. 2). This is a proof-of-principle demonstration that inexact models produced by *ab initio* prediction of structure from sequence can be used for the subsequent prediction of biochemical function. Of course, improvements in the method have to be made before such predictions can be done on a routine basis.

## Use of predicted structures from threading in protein-function prediction

At present, practical limitations preclude folding an entire genome of proteins using *ab initio* methods<sup>57</sup>. Threading is more appropriate for achieving the requisite high-throughput structure prediction. Thus, a standard threading algorithm<sup>58</sup> has been used to screen all

proteins in nine genomes for the disulfide-oxidoreductase active site described above.

First, sequences that aligned with the structures of known disulfide oxidoreductases were identified. Then, the structure was searched for matches to the active-site residues and geometry. For those sequences for which other homologs were available, a sequence-conservation profile was constructed<sup>23</sup>. If the putative active-site residues were not conserved in the sequence subfamily to which the protein belongs, that sequence was eliminated. Otherwise, the sequence is predicted to have the function.

Using this sequence-to-structure-to-function method, 99% of the proteins in the nine genomes that have known disulfide-oxidoreductase activity have been found. From 10% to 30% more functional predictions are made than by alternative sequence-based approaches; similar results are seen for the  $\alpha/\beta$ -hydrolases<sup>23</sup>. Surprisingly, in spite of the fact that threading algorithms have problems generating good sequence-to-structure alignments, active sites are often accurately aligned, even for very distant matches. This observation would agree with the above experimental results indicating that active sites are well conserved in protein structures.

Importantly, the false-positive rate when using structural information is much lower than that found using sequence-based approaches, as demonstrated by a detailed comparison of the FFF structural approach and the Blocks sequence-motif approach (N. Siew *et al.*, unpublished). In this study, the sequences in eight genomes, including *Bacillus subtilis*, were analysed for disulfide-oxidoreductase function using the disulfide-oxidoreductase FFF, the thioredoxin Block 00194 and the glutaredoxin Block 00195. If we assume that those sequences identified by both the FFF and Blocks are 'true positives', we find 13 such sequences in the *B. subtilis* genome.

There is no experimental evidence validating all of these 'true positives' and so they are more accurately termed 'consensus positives'. In order to find these 13 'consensus positive' sequences, the FFF hits seven false positives. On the other hand, Blocks hits 23 false positives (Fig. 3). It was previously suggested that the use of a functional requirement adds information to threading and reduces the number of false positives<sup>22</sup>. These data, including the data shown in Fig. 3, validate this claim on a genome-wide basis.

Of course, as no genome has had the function of all of its proteins experimentally annotated, it is impossible to know how many other proteins with the specified biochemical function were not properly identified. This is a critical question for researchers attempting to predict protein function. Experimental confirmation will be needed to validate this or any other method fully. This points out the need for closely coupling computational function-prediction algorithms with experiments.

#### Weaknesses of using the sequence-to-structure-to-function method of function prediction

Based on studies to date, the identification of enzymatic activity requires a model in which the backbone RMSD from native near the active sites is about 4–5 Å. Predicted models are better at describing the geometry in the core of the molecule than in the loops and so

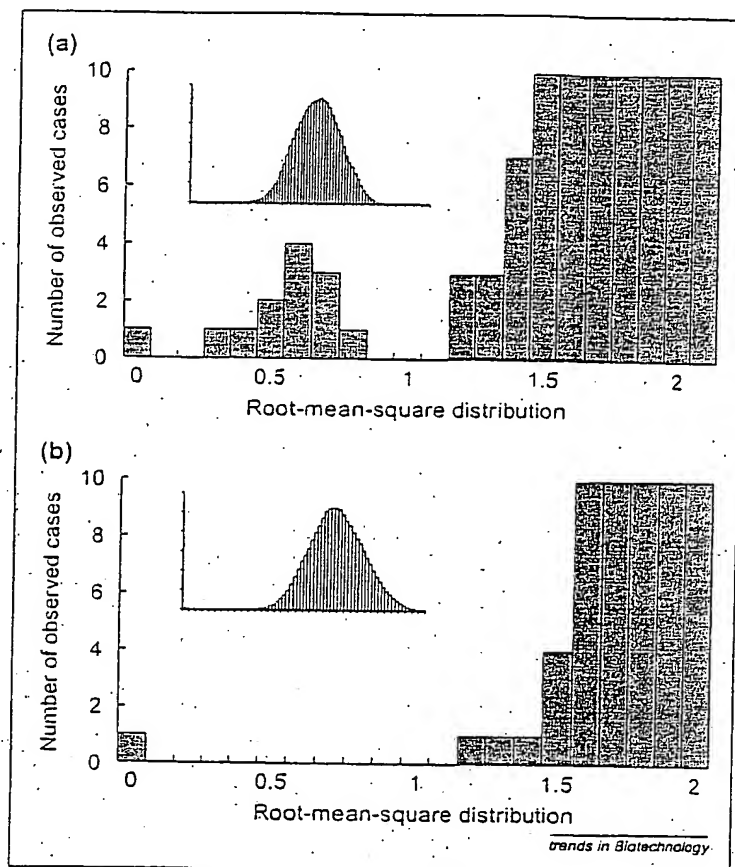


Figure 1

The distribution of root-mean-square distributions (RMSD) between the hydrolase catalytic triad and all other histidine-containing triads shows a bimodal distribution (a); by contrast, the RMSD between a randomly selected (non-catalytic) triad and all other histidine-containing triads has a unimodal distribution (b). The His-Ser-Asp catalytic triad in the protein-1 gpl (Rp2 lipase) (a) and a random histidine-containing triad from 4pga (glutaminase-asparaginase) (b) were structurally aligned to all His-containing triads in a database of 1037 proteins<sup>23</sup>. Actual  $\alpha/\beta$ -hydrolase active sites (a) and the 4pga site (b) are indicated by blue bars; other histidine triads that are not active sites are indicated by red bars. None of the sites found by matching to the 4pga were hydrolase active sites. Inset graphs show the full distribution.

predicting the function of a protein whose active site is in loops may be a problem. Also, the method can currently only be applied to enzyme active sites; substrate- and ligand-binding sites have not been identified using the inexact models. Techniques that will further refine inexact protein models will be quite useful in taking the protein analysis to the next step.

#### Conclusions

Although sequence-based approaches to protein-function prediction have proved to be very useful, alternatives are needed to assign the biochemical function of the 30–50% of proteins whose function cannot be assigned by any current methods. One emerging approach involves the sequence-to-structure-to-function paradigm. Such structures might be provided by structural-genomics projects or by structure-prediction algorithms. Functional assignment is made by screening the resulting structure against a library of structural descriptors for known active sites or binding regions.

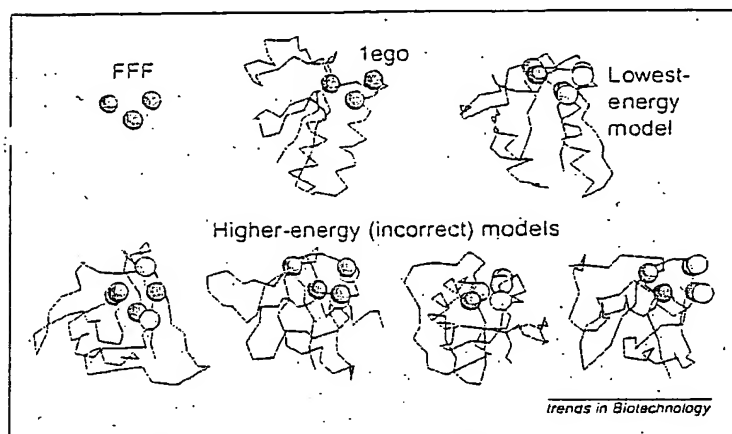


Figure 2

Application of the disulfide-oxidoreductase fuzzy functional form (FFF) to *ab initio* models of glutaredoxin created by the program MONSTER shows that the FFF can distinguish between correctly folded and misfolded (or higher-energy) models. The FFF is shown as two orange balls (representing the cysteines) and a blue ball (representing the proline). The protein models are shown as magenta wire models with the active-site cysteines and proline shown as yellow and cyan balls, respectively. The FFF clearly distinguishes the correct active site in the crystal structure of the glutaredoxin 1go and the correctly folded, lowest-energy model. The FFF does not match to the active sites of any of the higher energy, misfolded structures, four of which are shown here.

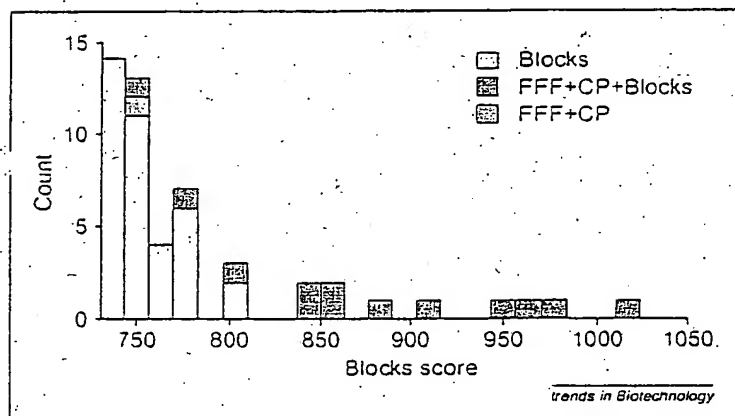


Figure 3

Analysis of the *Bacillus subtilis* genome using the thioredoxin Block 00194. The Blocks score (computed using the publicly available BLIMPS program) is plotted on the x axis and the number of sequences found in each scoring bin is plotted on the y axis. Those sequences identified as 'consensus positives' (identified by both the fuzzy functional form (FFF) and the Block) are shown as red bars. One additional sequence found by the FFF, which is likely to be a true positive, is shown as a blue bar. All other sequences; putative 'false positives', are shown as yellow bars. Using the Blocks score at which all 13 of the 'consensus positives' are found, 23 false positives are also found. In its analysis of the *B. subtilis* genome, the FFF identifies only seven false positives along with the same 13 'consensus positives' (data not shown).

Detailed descriptors will only work on the experimentally determined, high-quality structures. Ideally, however, the descriptors should work on both experimental structures and the cruder models provided by tertiary-structure-prediction algorithms.

The advantages of such an approach are that one need not establish an evolutionary relationship in order to assign function, that more than one function can be

assigned to a given protein [an issue of major importance, because proteins are multifunctional (Box 1)] and, ultimately, that having a structure can provide deeper insight into the biological mechanism of protein function and regulation. The disadvantages are that one needs to have the protein's structure before a function can be assigned and that the approach is limited to those functions associated with proteins with at least one solved structure, so that a functional-site descriptor can be constructed.

In this sense, structure-to-function assignment can be thought of as 'functional threading' – find the active-site match in a library of descriptors for known protein active sites. This is the first step in the long process of using structure to assign all levels of function, a goal that is made increasingly important with the emergence of structural genomics. Based on the progress to date, it is apparent that structure will play an important role in the post-genomic era of biology.

### Acknowledgment

We thank L. Zhang for producing the data in Box 2 and Fig. 1.

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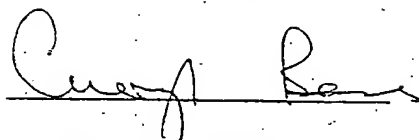
IN THE MATTER OF Australian Patent  
Application Serial No. 696764 by Human  
Genome Sciences, Inc.

-and-

IN THE MATTER OF Opposition thereto by  
Ludwig Institute for Cancer Research

THIS IS Exhibit PAWR-6  
referred to in the Statutory Declaration  
of Peter Adrian Walton Rogers  
made before me

DATED this 12<sup>th</sup> Day of November, 2001.



(Signature of Witness)

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# Interactions Between the Flk-1 Receptor, Vascular Endothelial Growth Factor, and Cell Surface Proteoglycan Identified with a Soluble Receptor Reagent

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Fetal liver kinase-1 (Flk-1) is a transmembrane tyrosine kinase that was identified in endothelial cells and populations of cells enriched in hematopoietic progenitors. To characterize the interaction of Flk-1 with potential ligands the receptor extracellular domain was genetically fused to an alkaline phosphatase (AP) tag. A soluble ligand for Flk-1 was identified in the supernatants of numerous mesenchymal cell lines by co-immunoprecipitation with the Flk1-AP fusion protein. This polypeptide was shown by N-terminal sequencing to be vascular endothelial growth factor (VEGF). Receptor-AP fusion proteins can thus be used to identify soluble ligands as well as transmembrane ligands, and this approach is therefore likely to be widely applicable to many types of orphan receptor. The Flk1-AP soluble receptor was also found to bind to cell surfaces, showing two apparent classes of binding site with different affinities. This interaction could be reconstructed by introducing a VEGF expression plasmid into cells. These results indicate that VEGF presented at the cell surface can bind to the Flk-1 receptor, and could mediate a direct cell-cell interaction. The Flk1-AP fusion protein was also found to bind heparin, implying that ligand binding by the Flk-1 receptor may involve a three way interaction between the Flk-1 receptor, VEGF, and heparin-like cell surface proteoglycans.

KEYWORDS: receptor tyrosine kinase, heparin, alkaline phosphatase

## INTRODUCTION

Cell surface receptors with an intracellular tyrosine kinase domain have powerful effects on proliferation and other aspects of cell behavior. When activated by mutation they can act as potent oncogenes, and they have important roles in normal physiology and development (Schlessinger and Ullrich, 1992). In addition to the receptor tyrosine kinases with known ligands, many additional receptor-like tyrosine kinases without known ligands have been identified, mostly by approaches based on the sequence conservation of the enzymatic tyrosine kinase domain. More than twenty of these orphan receptors are currently without known ligands, and it is likely that the identification of those ligands will

make an important contribution to our understanding of cell-cell signaling.

The Flk-1 receptor was identified by polymerase chain reaction of mRNA from populations of mouse fetal liver cells highly enriched for primitive hematopoietic progenitors (Matthews et al., 1991). The Flk-1 receptor, and an apparent human homolog, KDR, was also found to be expressed in vascular endothelial cells (Terman et al., 1991; Millauer et al., 1993; Quinn et al., 1993). Structurally, the Flk-1 receptor has an extracellular region containing seven immunoglobulin-like domains, placing it in a subfamily with two other receptors that show close sequence homology. Those receptors are flt, which was shown to be a receptor for vascular endothelial growth factor (de Vries et al., 1992), and flt-4 (Aprelikova et al., 1992; Galland et al., 1992), a receptor still without a known ligand.

One approach to identifying the ligands of receptor tyrosine kinases is to use the receptor

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extracellular domain as a soluble affinity reagent. We have previously described the use of a soluble form of the c-kit receptor for that purpose (Flanagan and Leder, 1990). The receptor extracellular domain was fused to placental alkaline phosphatase, providing the molecule with a tag that binds to available antibodies and also has an intrinsic enzyme activity that can be easily and sensitively traced. This reagent was used to detect the kit ligand as a transmembrane molecule expressed at the cell surface (Flanagan and Leder, 1990). Similar approaches based on soluble receptor fusion proteins have now also been used to identify other transmembrane ligands at the cell surface (for example, Armitage et al., 1992; Lyman et al., 1993).

Here we have applied the soluble receptor approach to the Flk-1 receptor and shown that this approach can be used to identify not only transmembrane ligands, but also ligands that are matrix-associated or soluble. The Flk-1 receptor was found by this approach to bind with high affinity to VEGF, an interaction also described by others (Mullauer et al., 1993; Quinn et al., 1993). The interaction was detectable in solution and also when VEGF was present at cell surfaces, where it is bound via cell surface proteoglycan. In addition, the soluble Flk-1 receptor itself was found to bind to heparin-agarose. These results suggest that a three-way functional complex may be formed between the Flk-1 receptor, VEGF, and specific heparin-like molecules at the surface of the ligand-presenting cell.

## METHODS

### Production of Flk1-AP Fusion Protein

The Flk-1 extracellular region cDNA was amplified by polymerase chain reaction to create a HindIII-BamHI fragment, which was inserted into HindIII-BglII cut APTag-1 vector (Flanagan and Leder, 1990). The resulting Flk1-AP fusion plasmid encodes the entire extracellular domain of Flk-1 joined at Glu-762, via a four amino acid linker (Gly-Ser-Ser-Gly), to the distinctively heat-stable secreted human placental alkaline phosphatase. A plasmid with the same vector sequences encoding unfused secreted alkaline phosphatase (SEAP) (Berger et al., 1988) was also produced for use as a control. Plasmids were linearized with ClaI and transfected with the marker plasmid pSV7neo into NIH-3T3 cells by calcium phosphate precipitation. One day after

transfection, cells were distributed into 96-well plates and selected with 400  $\mu$ g/ml G418. After 2 weeks, approximately 100 clones were screened for secretion of alkaline phosphatase activity by a colorimetric assay as described (Flanagan and Leder, 1990), except that L-homoarginine was omitted from all alkaline phosphatase assays here. Alkaline phosphatase activities are expressed here as OD units per hour (OD/hr), indicating the rate of hydrolysis of the chromogenic substrate p-nitrophenyl phosphate under the conditions used. One picomole of alkaline phosphatase protein corresponded to an activity of approximately 30 OD/hr.

### Co-immunoprecipitation with the Flk1-AP Fusion Protein

For analysis of  $^{35}$ S-labeled proteins in supernatants, cells in 10 cm plates were rinsed twice in methionine-free DMEM, then incubated with 200  $\mu$ Ci/ml  $^{35}$ S protein labeling mix (New England Nuclear, NEG-072) in 4 ml of methionine-free DMEM containing 10% dialyzed calf serum. After 8 hr, supernatants were taken and concentrated 10 fold by ultrafiltration. 200  $\mu$ l of concentrated supernatant was incubated for 90 min at room temperature with an equal volume of conditioned medium containing approximately 5  $\mu$ g/ml Flk1-AP fusion protein. The Flk1-AP fusion protein was then immunoprecipitated by incubating on a rotator for 60 min with CNBr-Sepharose beads coupled to excess monoclonal antibody against human placental alkaline phosphatase (Cat. no. MIA 1801, Medix Biotech Inc., Foster City, CA) and washing the beads six times in modified RIPA buffer (0.5% NP40, 0.5% NaDOC, 0.025% SDS, 144 mM NaCl, 20 mM Tris-HCl pH 8.0).  $^{35}$ S-labeled proteins were separated on 15% SDS-polyacrylamide gels, which were then fixed, treated with Enlightening (New England Nuclear), dried, and exposed to X-ray film.

To prepare proteins for microsequencing, 200  $\mu$ l of CNBr-Sepharose was conjugated with 400  $\mu$ g anti-AP antibody and was then incubated with a saturating amount of Flk1-AP conditioned medium for 1 hr at room temperature. The beads were then washed and treated with the crosslinking agent dimethylpimelimidate (Harlow and Lane, 1988). BMS-12 cells were grown in DMEM containing 1% bovine calf serum for 3 days. One liter of conditioned medium was collected and concentrated to

30 ml by ultrafiltration and was incubated with the 200  $\mu$ l of Flk1-AP conjugated beads. After 1 hr at room temperature the beads were packed into a column, washed with 4 ml of modified RIPA buffer, 1 ml of 10 mM sodium phosphate pH 6.8, and proteins were then eluted with 1 ml of 100 mM glycine, pH 2.5. The eluted sample was concentrated by TCA precipitation, run on a 15% SDS-polyacrylamide gel, and transferred to a PVDF membrane (Matsudaira, 1987). The band of interest was cut out and gas-phase protein sequencing was performed as described (Tempst and Riviere, 1989).

#### Quantitative Assays of Flk1-AP Binding to Cell Surfaces or to Heparin Sepharose

Cell surface binding of Flk1-AP was measured essentially as described previously for Kit-AP (Flanagan and Leder, 1990). Plates of cells were washed with HBHA buffer (Hank's balanced salt solution with 1 mg/ml bovine serum albumin, 20 mM HEPES pH 7.0, 0.1%  $\text{NaN}_3$ ) and then incubated for 90 min at room temperature with conditioned medium containing Flk1-AP fusion protein or SEAP protein as a control. For some experiments the conditioned medium was diluted with

HBHA buffer. The cells were then rinsed six times with HBHA buffer, lysed, and assayed for alkaline phosphatase activity colorimetrically as described (Flanagan and Leder, 1990). Scatchard analyses of cell surface binding data were performed with the LIGAND program (Munson and Rodbard, 1980).

To test the effect of heparinase on binding of Flk1-AP to the cell surface, cells were incubated in DMEM without serum for 1 hr at 37°C with 0.25 units/ml of heparinase (a gift from Ibex Technologies, Montreal, Canada). To test binding of Flk1-AP to heparin-Sepharose, conditioned medium containing Flk1-AP or SEAP was diluted into 10-ml of 20 mM HEPES pH 7.0, 150 mM NaCl and was loaded onto a 1 ml heparin Sepharose FPLC column (HiTrap column, Pharmacia) by recirculating overnight. Salt gradient elution was performed at a flow rate of 0.5 ml/min with collection of 1 ml fractions.

#### RESULTS

The Flk1-AP soluble receptor fusion protein used in these studies is illustrated in Fig. 1A. When immunoprecipitated from the supernatant of transfected

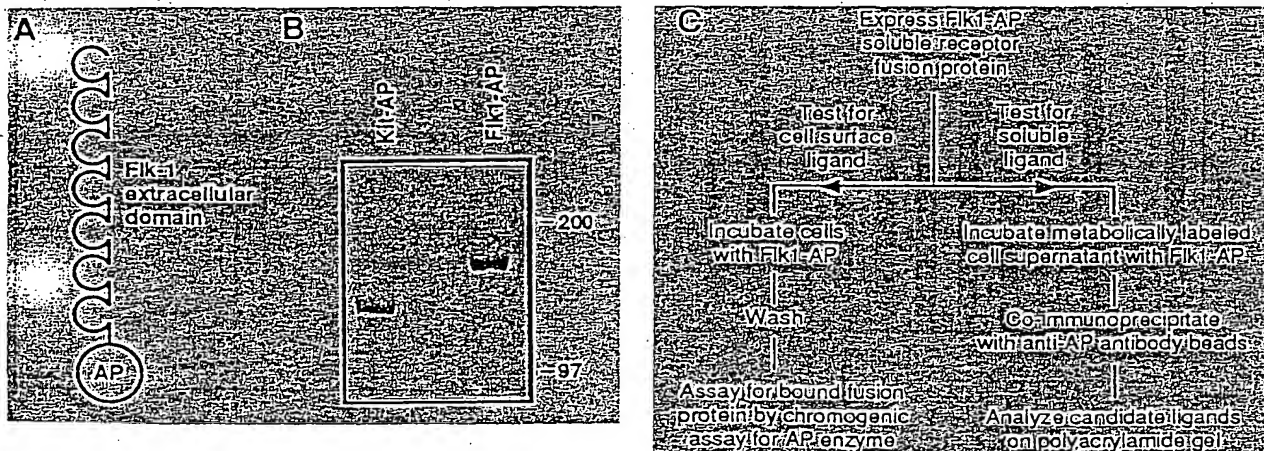


FIGURE 1. A soluble receptor affinity reagent used to screen for cell surface or soluble ligands. (A) The structure of the Flk1-AP soluble receptor fusion protein, consisting of the seven immunoglobulin-like domains of the Flk-1 extracellular region fused to a human placental alkaline phosphatase tag. (B) The Flk1-AP fusion reagent was produced by transfected NIH-3T3 cells as a single major polypeptide of the expected molecular weight. The supernatant of a transfected clone expressing high levels of alkaline phosphatase activity was immunoprecipitated with anti-AP beads and analyzed by electrophoresis on a 6% polyacrylamide gel followed by Coomassie blue staining. A Kit-AP fusion protein is shown for comparison (Flanagan and Leder, 1990). (C) Illustrates the strategy used to perform an initial screen of cell lines for either a cell surface ligand or a soluble ligand. To screen for a cell surface ligand (left side) the cells were treated with Flk1-AP fusion protein, washed and then tested for binding of the soluble receptor by a simple colorimetric assay for bound AP activity. To test for soluble ligand (right side) metabolically labeled supernatants from candidate cell lines were mixed with supernatant containing Flk1-AP fusion protein. The fusion protein, with any bound ligand, was then immunoprecipitated with anti-AP beads, and radiolabeled proteins were analyzed by gel electrophoresis.

cells, the fusion protein appears as a single prominent band with the expected apparent molecular weight of approximately 170 kDa (Fig. 1B). This fusion protein retained alkaline phosphatase enzyme activity, with a specific activity similar to that reported previously (Flanagan and Leder, 1990). Individual clones of transfected cells selected for secretion of high alkaline phosphatase activity produced approximately 5  $\mu\text{g}/\text{ml}$  of fusion protein in the supernatant.

The strategy used to screen cell lines for production of a Flk-1 ligand is summarized in Fig. 1C. As Flk-1 was implicated as a receptor that might function in hematopoietic progenitors and endothelial cells, we focused our initial ligand search on mesenchymal cell lines such as bone marrow stromal cells and embryonic fibroblasts, on the basis that such cells might be expected to support the growth of the receptor-bearing cell types. Figure 2 shows the result of a screen for cell surface binding to thirteen cell lines, including ten mouse bone marrow stromal lines (BMS and BMSC lines), one mouse embryonic fibroblast line (STO), one rat liver cell

line (BRL 3A) and one mouse macrophage line (P388D1). The cells were treated with Flk1-AP to test for the presence of a candidate ligand and also with unfused SEAP as a control for background binding. In repeated experiments, each of the cell lines tested except P388D1 and BRL 3A showed cell surface binding of Flk1-AP that was several times higher than the SEAP control. Representative results are shown in Fig. 2.

As a biologically significant receptor-ligand interaction is expected to have a reasonably high affinity, a Scatchard analysis of the cell surface binding was performed by carrying out the binding assay with varying amounts of Flk1-AP. The binding data give a nonlinear Scatchard plot consistent with binding to two sites of different affinities on the cell surface (Fig. 3). This is in contrast to other AP tagged soluble receptors, such as the c-kit receptor, which give linear Scatchard plots when tested for binding to their cell surface ligands (Flanagan and Leder, 1990; and unpublished data). The dissociation constants for Flk1-AP binding to the cell surface calculated from repeated experiments were approximately  $10^{-10}$  M for the higher affinity site and

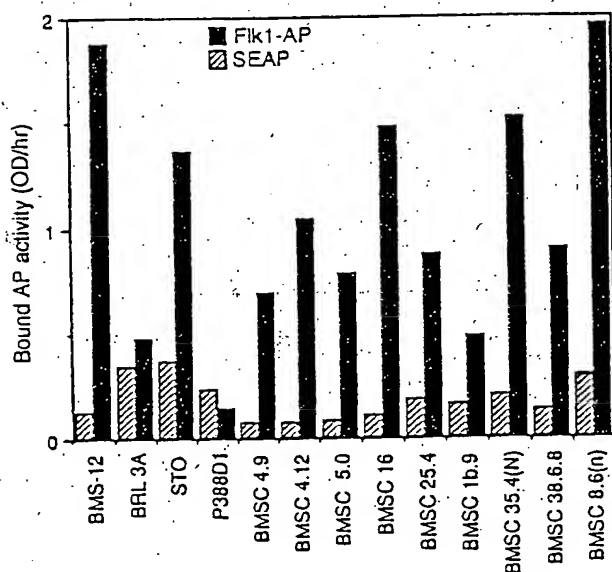


FIGURE 2. Binding of Flk1-AP soluble affinity reagent to the surfaces of cell lines. Cell lines in 10 cm dishes were incubated with 4 ml of conditioned medium containing Flk1-AP or SEAP as a control, each at 600 OD/hr per ml, for 1 hr at room temperature, then the cells were washed and analyzed for bound alkaline phosphatase activity. Cell lines denoted BMS or BMSC are mouse bone marrow stromal lines (M.-K.C., J.G.F., and N. Weich and W. Benjamin, Hoffman LaRoche Inc.), BRL 3A is a rat liver line, STO is a mouse embryo fibroblast line, and P388D1 is a mouse macrophage line.

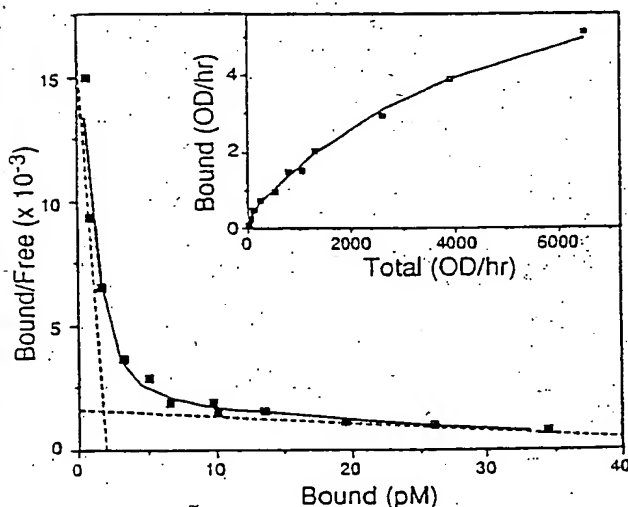


FIGURE 3. Scatchard analysis of Flk1-AP binding to the surface of BMS-12 cells. BMS-12 hematopoietic stromal cells in 10 cm plates were incubated with varying concentrations of Flk1-AP fusion protein in 4 ml of medium, and were then washed and assayed for bound alkaline phosphatase activity. The inset graph shows the measured alkaline phosphatase activities. The same data are shown as a Scatchard plot with a curve calculated for two cell surface binding sites of different affinities. The results of this experiment indicate approximately 170,000 sites per cell with a dissociation constant of  $3.5 \times 10^{-8}$  M and 6000 sites per cell with a dissociation constant of  $1.3 \times 10^{-10}$  M.



approximately  $10^{-8}$  M for the lower affinity site, and the numbers of sites per cell were approximately 5000 and 100,000 respectively (Fig. 3). The dissociation constant of the higher affinity site is similar to recent estimates of the affinity of  $^{125}$ I-labeled VEGF binding to Flk-1 receptor expressed on cell surfaces (Millauer et al., 1993; Quinn et al., 1993).

In parallel with the screen for cell surface ligands, we also screened cell lines for secretion of soluble Flk-1 ligands by a co-immunoprecipitation procedure (Fig. 1C). Two polypeptides with apparent molecular weights of approximately 19 and 23 kDa were detected prominently in supernatants of all the cell lines tested, including those shown in Fig. 4 as well as the 9 additional hematopoietic stromal cell lines described in Fig. 2. These bands were absent

from controls where unfused SEAP was substituted for the Flk1-AP fusion protein (Fig. 4A). Experiments where the amount of Flk1-AP fusion protein in the co-immunoprecipitation reaction was varied indicated that the affinity of the soluble receptor for these polypeptides was approximately in the nanomolar range, consistent with a biologically significant receptor-ligand interaction and with the results obtained from the cell surface binding experiments.

The yield of the candidate Flk-1 ligand polypeptides in co-immunoprecipitation experiments was several nanograms per ml of supernatant, as judged by silver staining of gels (data not shown). This implied that amino acid sequence information could be obtained from a moderate amount of conditioned

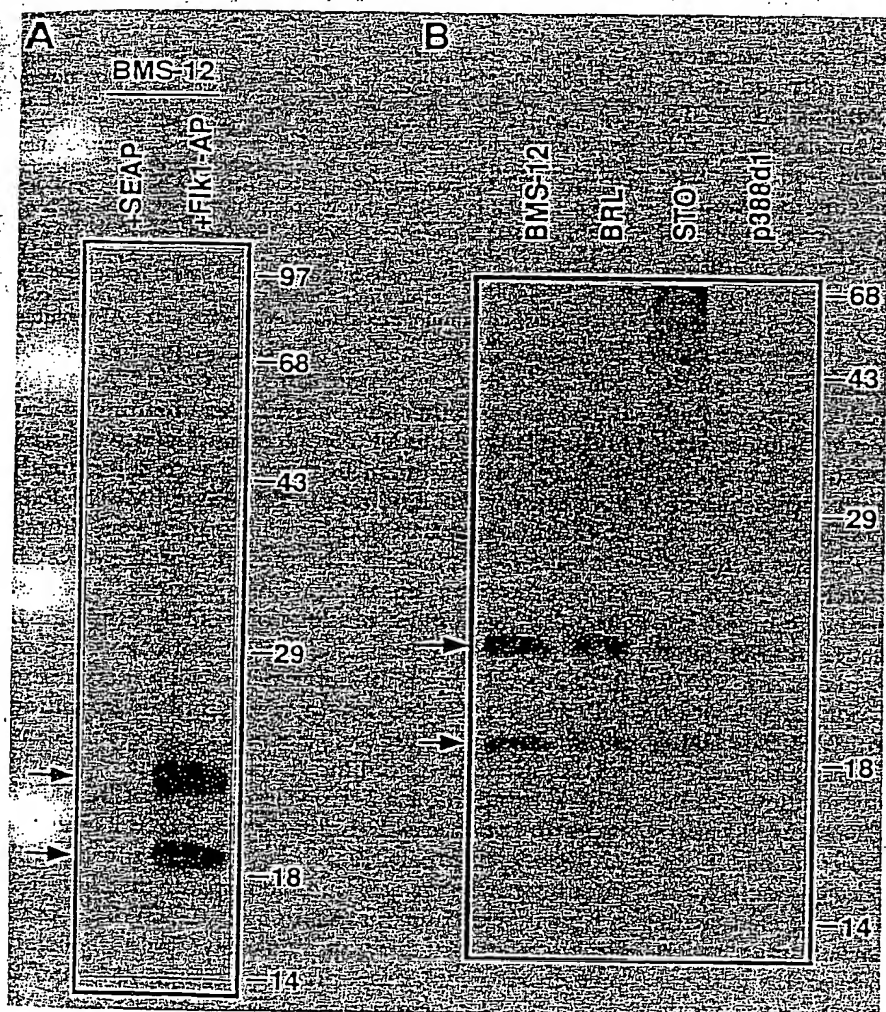


FIGURE 4. Co-immunoprecipitation of candidate ligand polypeptides with Flk1-AP. (A) BMS-12 bone marrow stromal cells were labeled with  $^{35}$ S-methionine, then the supernatant was collected and treated with Flk1-AP fusion protein or SEAP as a control, followed by immunoprecipitation with anti-AP beads. Labeled proteins were then analyzed on a 15% polyacrylamide gel. (B) Supernatants from a variety of cell lines were co-immunoprecipitated with Flk1-AP, including a bone marrow stromal line (BMS-12), a liver cell line (BRL 3A), an embryonic fibroblast (STO), and a macrophage line (P388D1).

medium. One liter of supernatant from the BMS-12 cell line was therefore concentrated and co-immunoprecipitated, yielding approximately 2  $\mu$ g of each of the candidate ligand polypeptides. After blotting onto a PVDF membrane, the 23 kDa band was subjected to N-terminal peptide microsequencing. The first nine amino acids of the resulting sequence were found to match the N-terminal sequence (APTTEGEQK) predicted from the cDNA of murine VEGF (Breier et al., 1992; Claffey et al., 1992).

To confirm binding of VEGF to the Flk-1 receptor, murine VEGF cDNAs were isolated by polymerase chain reaction (PCR). BMS-12 and two other lines (STO and P388D1) that express the 19 and 23 kDa co-immunoprecipitated polypeptides (Fig. 4) were tested by PCR and were each found to yield two prominent amplified bands visible by agarose gel electrophoresis (data not shown). Nucleotide sequencing of the cDNAs in these bands indicated that they correspond to previously described alternatively spliced forms of VEGF cDNA, called VEGF-1 and VEGF-2 (Breier et al., 1992; Claffey et al., 1992). When expressed in COS cells, both of these cDNAs yielded polypeptides in the supernatant that co-immunoprecipitate with Flk1-AP fusion protein (Fig. 5). The apparent molecular weights yielded by the VEGF-1 and VEGF-2 constructs were approximately 23 and 19 kDa respectively, corresponding in size to the two bands precipitated from BMS-12 (Fig. 5). These results further confirm that the 19 and 23 kDa polypeptides co-immunoprecipitated from BMS-12 and other cell lines are almost certainly VEGF polypeptides. The results also show that the VEGF-1 and VEGF-2 polypeptides both bind to the Flk-1 receptor.

It is known that VEGF can attach to the surface of expressing cells, and that this attachment can be eliminated by heparinase treatment (Ferrara et al., 1992). It therefore seemed possible that the binding of Flk1-AP to cell surfaces is mediated by VEGF associated with cell surface proteoglycans. To test this, COS cells were transfected with VEGF-1 cDNA and tested for Flk1-AP binding. Untransfected COS cells showed low levels of Flk1-AP binding, while cells expressing transfected VEGF showed much higher levels of binding (Fig. 6). Like the data from the BMS-12 stromal cell line, the binding data from the transfected COS cells are consistent with binding to two sites of different affinities, with apparent dissociation constants comparable to those measured for BMS-12 (Fig. 6).



FIGURE 5. Co-immunoprecipitation of VEGF polypeptides with Flk1-AP. Two alternatively spliced forms of VEGF cDNA, VEGF-1 and VEGF-2, were transfected into COS cells by the DEAE dextran method as described (Flanagan et al., 1991). BMS-12 cells and transfected COS cells were metabolically labeled with  $^{35}$ S-methionine, then the supernatants were collected and co-immunoprecipitated with the Flk1-AP soluble receptor fusion protein.

To assess further the nature of the binding of the Flk1-AP protein to VEGF on cell surfaces, we tested the effect of treatment of the cells with salt or with heparinase. The binding of Flk1-AP to BMS-12 cells or to transfected COS cells was found to be almost completely inhibited by the presence of 0.6 M NaCl. Pretreatment of the cells with heparinase also removed most of the binding of Flk1-AP (Fig. 7A). These results are consistent with an involvement of ionic interactions with heparin-like molecules at the cell surface. These interactions probably include binding of VEGF to cell surface proteoglycans, as VEGF is known to bind heparin (Ferrara et al., 1992). In addition it seemed possible that the Flk-1 receptor might itself bind directly to cell surface proteoglycans, particularly in view of the recent demonstration of an interaction of the FGF-R1 receptor with heparin (Kan et al., 1993). This possibility was tested by applying the Flk1-AP fusion protein to a heparin-Sepharose column. At pH 7.0 and a NaCl concentration of 150 mM, the Flk1-AP

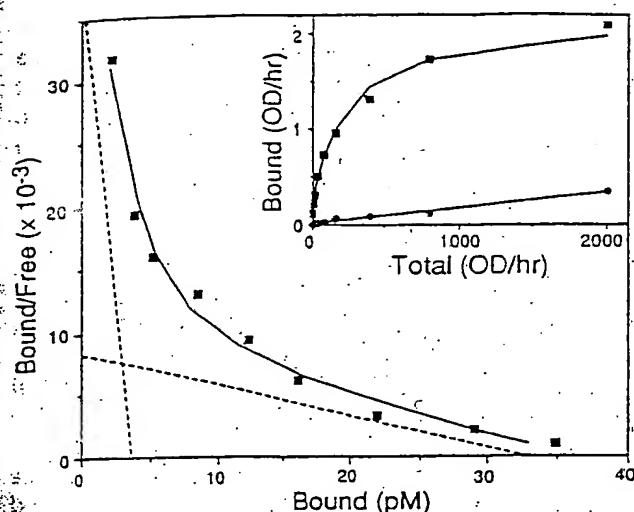


FIGURE 6. Scatchard analysis of Flk1-AP binding to the surface of COS cells transfected with VEGF cDNA. COS cells in 6 cm plates were treated with varying concentrations of Flk1-AP fusion protein in 2 ml of medium. The cells were then washed and assayed for bound alkaline phosphatase activity. The inset graph shows the measured alkaline phosphatase activities for cells transfected with VEGF-1 cDNA (squares) or vector alone (circles). The same data are shown as a Scatchard plot with a curve calculated for two cell surface binding sites of different affinities. The results of this experiment indicate approximately 135,000 sites per cell with a dissociation constant of  $1.1 \times 10^{-8}$  M and 29,000 sites per cell with a dissociation constant of  $3.1 \times 10^{-10}$  M.

fusion protein bound to the heparin column (Fig. 7B). SEAP alone did not bind effectively (Fig. 7B), consistent with the low isoelectric point of placental alkaline phosphatase which would give it a net negative charge at pH 7.0. The Flk1-AP fusion protein was also found not to bind to unconjugated Sepharose (data not shown) indicating that it binds to the heparin moiety of the heparin-Sepharose matrix. Bound Flk1-AP fusion protein was eluted from the heparin-Sepharose column by NaCl concentrations of approximately 0.3 M (Fig. 7B). The interaction of Flk1-AP with the heparin column may be direct or might be mediated by other molecules present in the conditioned medium. However, it is unlikely that the Flk1-AP binding is mediated by VEGF, because the Flk1-AP concentration in the conditioned medium is much higher than the expected concentration of VEGF. The results therefore suggest that binding of the Flk-1 receptor extracellular domain to heparin-like molecules may be involved in the formation of a three-way functional complex between the Flk-1 receptor, heparin-like components and VEGF.

## DISCUSSION

Flk-1 is one of a large number of receptor tyrosine kinases that were identified by the nucleotide sequence conservation of the kinase domain, but initially had no known ligands. We and others have previously used soluble versions of cell surface receptors to identify ligands that are transmembrane molecules (for example, Flanagan and Leder, 1990; Lyman et al., 1993). However, it has been less clear whether the soluble receptor approach could also be generally applied to ligands that are soluble. Here we have used a receptor-AP fusion protein to identify a soluble ligand for the Flk-1 receptor. The Flk1-AP reagent was used in a co-immunoprecipitation procedure to identify a candidate ligand in the supernatants of numerous mesenchymal cell lines. Co-immunoprecipitation from moderate amounts of supernatant allowed the isolation of a sufficient yield of the ligand for peptide microsequencing, showing that this ligand is VEGF. This growth factor was also shown by others to bind Flk-1 or its human homolog KDR, and was found to activate Flk-1 kinase activity (Terman et al., 1992; Millauer et al., 1993; Quinn et al., 1993). These results support the idea that the soluble receptor affinity approach is likely to be of general utility for the identification and characterization of a wide variety of different types of ligand, whether they are transmembrane, matrix-associated or soluble.

Placental alkaline phosphatase serves as a useful fusion protein tag. The availability of antibodies against placental alkaline phosphatase makes it straightforward to use procedures such as co-immunoprecipitation. Moreover the marker enzyme activity of the tag allows the fusion protein to be traced quantitatively by simple chromogenic assays without the necessity of purification, radioactive labeling, or the use of secondary reagents. We find that detection using the enzyme activity of AP fusion proteins is compatible with a variety of applications including quantitative receptor-ligand binding studies, *in situ* staining for ligands, and library screening. The sensitivity of detection can be at least comparable to other methods, such as the use of purified and  $^{125}$ I labeled reagents (Flanagan and Leder, 1990; Flanagan et al., 1991; Ornitz et al., 1992, this paper, and unpublished data).

VEGF was originally discovered as a secreted polypeptide that affects endothelial cell growth and vascular permeability, and it has been implicated as an important factor in angiogenesis associated with

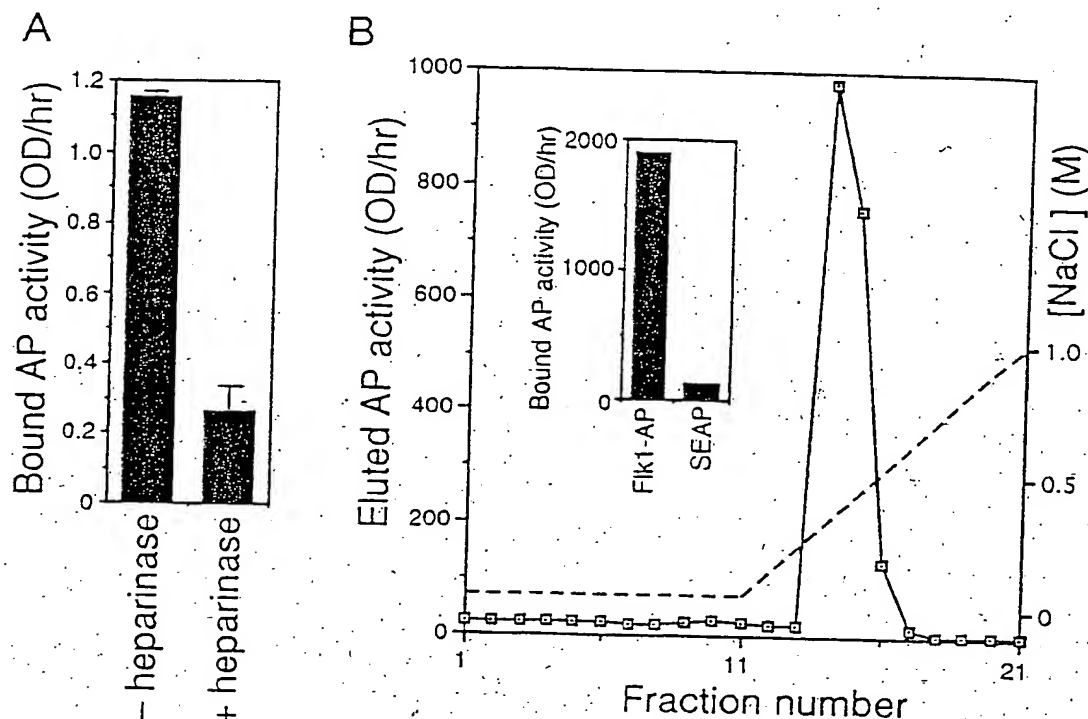


FIGURE 7. Role of heparin-like molecules in the interactions of Flk1-AP. (A) Effect of heparinase on the binding of Flk1-AP to VEGF on cell surfaces. COS cells transfected with VEGF-1 cDNA were incubated with or without heparinase, then were treated with Flk1-AP (500 OD/hr/ml), washed and assayed for bound AP activity. Each bar indicates the mean of two determinations, and the error bars indicate the difference between the two. (B) Binding of Flk1-AP to heparin-Sepharose. Approximately 2500 OD/hr (12.5  $\mu$ g) of Flk1-AP fusion protein or the same activity of SEAP protein was loaded onto a 1 ml heparin-Sepharose FPLC column. The inset histogram shows total binding of Flk1-AP or SEAP. A salt gradient elution profile of Flk1-AP is also shown (solid line). After Flk1-AP binding the column was washed with 10 ml of buffer containing 50 mM NaCl, and bound proteins were then eluted with a gradient of NaCl (broken line).

both tumor formation and normal development (Folkman and Klagsbrun, 1987; Breier et al., 1992; Claffey et al., 1992; Ferrara et al., 1992; Plate et al., 1992; Shweiki et al., 1992; Kim et al., 1993; Millauer et al., 1993; Quinn et al., 1993; Millauer et al., 1994). In mice, two major alternatively spliced forms of VEGF mRNA and an additional minor form have been identified (Breier et al., 1992; Claffey et al., 1992). In this study we found that Flk-1 binds the protein products of both major alternative spliced forms of VEGF, VEGF-1 and VEGF-2. As Flk-1 is a receptor for VEGF and is expressed in endothelial cells or their progenitors from early stages of development, it is likely that the interaction of VEGF with the Flk-1 receptor has an important role in the control of angiogenesis (Millauer et al., 1993; Quinn et al., 1993; Millauer et al., 1994). The expression of Flk-1 in populations of cells highly enriched for primitive hematopoietic progenitors is also sugges-

tive of possible roles for this receptor in hematopoiesis (Matthews et al., 1991), and could be consistent with the possible existence in the embryonic blood islands of a common progenitor for endothelial cells and hematopoietic stem cells (Risau, 1991).

Receptor-ligand interactions are typically studied at the surface of the receptor-bearing cell, although the interactions of receptors, growth factors and accessory molecules at the surface of the ligand-presenting cell may also be an important determinant of biological activity. The use of soluble receptor fusion proteins, as described here, allows a characterization of such interactions. We find that the soluble Flk-1 receptor binds VEGF-1 on the surfaces of expressing cells, without treatment to release the factor into solution. These results indicate that when VEGF is associated with proteoglycans or other molecules at the surface of the expressing cell, it is not sequestered from direct



binding to its receptor and is thus likely to be capable of mediating a direct cell-cell interaction. Interestingly, the binding of soluble Flk-1 to VEGF on the cell surface produces a non-linear Scatchard plot consistent with binding to two classes of site with different affinities. This could be due to an involvement of heparin-like molecules in the interaction. For example one model would be that the lower affinity site could represent a simple interaction between VEGF and Flk-1 only, while the higher affinity site could be produced by a further stabilization of this complex by heparin-like molecules interacting with both VEGF and Flk-1. Further support for this model comes from the finding that the soluble Flk-1 receptor can itself bind to heparin, suggesting the possibility of a direct interaction between the Flk-1 receptor and heparin-like molecules at the cell surface or in extracellular matrix. This model is also consistent with a recent report showing that the interaction of soluble VEGF and Flk-1 can be modulated by soluble heparin (Tessier et al., 1994).

Our results suggest that there may be some cell type specificity in the ability to present VEGF. For example, the P388D1 and BRL 3A cell lines were found to express VEGF polypeptides, but little or no Flk1-AP fusion protein bound to the surface of those cell lines. Specific cell surface proteoglycans may therefore be required to present VEGF on the cell surface. In this regard, it is of interest that the P388D1 line was recently shown to express a specific subset of cell surface proteoglycans (Yeaman and Rapraeger, 1993). Specific interactions with accessory proteoglycans might play an important role in modulating the activity of VEGF and could represent potential targets for therapeutic intervention.

In several respects VEGF is similar to basic fibroblast growth factor. They are both heparin binding growth factors and have angiogenic activity *in vitro* and *in vivo* (Folkman and Klagsbrun, 1987; Ferrara et al., 1992; Plate et al., 1992; Shweiki et al., 1992; Kim et al., 1993; Millauer et al., 1994). Heparin-like molecules are known to promote high affinity functional binding between both factors and their receptors (Klagsbrun and Baird, 1991; Rapraeger et al., 1991; Yayon et al., 1991; Bernfield et al., 1992; Gitay-Goren et al., 1992; Ornitz et al., 1992). A receptor-heparin interaction was observed here for Flk-1, and has also been reported for the fibroblast growth factor receptor FGF-R1 (Kan et al., 1993). The specific spatial localization of angiogenic factors

on the cell surface or extracellular matrix and the effect of such interactions on the ability of their receptors to recognize these factors may play important roles in the control of angiogenesis.

## ACKNOWLEDGMENTS

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*Patents Act 1990*

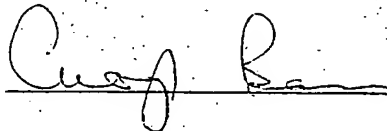
IN THE MATTER OF Australian Patent  
Application Serial No. 696764 by Human  
Genome Sciences, Inc.

-and-

IN THE MATTER OF Opposition thereto by  
Ludwig Institute for Cancer Research

THIS IS Exhibit PAWR-7  
referred to in the Statutory Declaration  
of Peter Adrian Walton Rogers  
made before me

DATED this 12<sup>th</sup> Day of November, 2001



(Signature of Witness)

Medical Practitioner

## Heparin Modulates the Interaction of VEGF<sub>165</sub> with Soluble and Cell Associated *flk-1* Receptors\*

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The 165-amino acid form of vascular endothelial growth factor (VEGF<sub>165</sub>) is a mitogen for vascular endothelial cells and a potent angiogenic factor. Expression of a chimeric receptor containing the extracellular domain of the *flk-1* receptor fused to the transmembrane and intracellular domains of the human *c-fms* receptor in NIH-3T3 cells, resulted in the appearance of high affinity binding sites for <sup>125</sup>I-VEGF<sub>165</sub> on transfected cells. The binding of <sup>125</sup>I-VEGF<sub>165</sub> to the *flk-1/fms* chimeric receptor of the transfected cells as well as the VEGF<sub>165</sub> induced autophosphorylation of the chimeric receptors were inhibited in the presence of low concentrations of heparin (1–10 µg/ml). In contrast, similar concentrations of heparin potentiated the binding of <sup>125</sup>I-VEGF<sub>165</sub> to the endogenous VEGF receptors of the transfected cells, indicating that to some extent, the effect of heparin on <sup>125</sup>I-VEGF<sub>165</sub> binding is receptor type-dependent.

A soluble fusion protein containing the extracellular domain of *flk-1* fused to alkaline phosphatase (*flk-1*/SEAP) was used to study the effects of heparin on the binding of <sup>125</sup>I-VEGF<sub>165</sub> to *flk-1* in a cell-free environment. The fusion protein specifically inhibited VEGF<sub>165</sub> induced proliferation of vascular endothelial cells, but bound <sup>125</sup>I-VEGF<sub>165</sub> inefficiently in the absence of heparin. Addition of low concentrations of heparin or heparan sulfate (0.1–1 µg/ml) resulted in a strong potentiation of <sup>125</sup>I-VEGF<sub>165</sub> binding, whereas higher heparin or heparan sulfate concentrations inhibited the binding. The effect of heparin on the binding of <sup>125</sup>I-VEGF<sub>165</sub> to *flk-1*/SEAP could not be mimicked by desulfated heparin or by chondroitin sulfate. Both bFGF and aFGF inhibited the binding when low concentrations of heparin were added to the binding reaction. However, higher concentrations of heparin abolished the inhibition, indicating that the inhibition is probably caused by competition for available heparin. Taken as a whole, these results indicate that heparin-like molecules regulate the binding of VEGF<sub>165</sub> to its receptors in complex ways which depend on the heparin binding properties of VEGF<sub>165</sub>, on the specific VEGF receptor type involved, and on the amount and composition of heparin-like molecules that are present on the cell surface of VEGF receptor containing cells.

Vascular endothelial growth factor (VEGF)<sup>1</sup> is a secreted heparin-binding glycoprotein that displays some structural homology with PDGF. Five forms of human VEGF mRNA encoding VEGF proteins of 121, 145, 165, 189, and 206 amino acids are produced from a single gene as a result of alternative splicing (1, 2). The best characterized VEGF species is the 165-amino acid long form (VEGF<sub>165</sub>). The active form of VEGF<sub>165</sub> is a homodimer of 47 kDa (1, 3) that induces angiogenesis and blood vessel permeabilization *in vivo* and displays a mitogenic activity that seems to be restricted to vascular endothelial cells (1, 4–8). Several recent reports indicate that VEGF may play an important role in the process of tumor angiogenesis (9–11). VEGF<sub>165</sub> binds to specific cell surface receptors which are found on vascular endothelial cells, and on several types of non-endothelial cells such as NIH-3T3 cells and melanoma cells which do not seem to respond to VEGF<sub>165</sub> with a mitogenic response (12–16). Cell surface-associated heparin-like molecules are required for the interaction of VEGF<sub>165</sub> with the three VEGF receptor types observed on vascular endothelial cells (15) and with the two receptor types present in WW94 melanoma cells (16).

The protein encoded by the *flt* gene was recently reported to be a VEGF receptor (17, 18), belonging to the PDGF receptor subfamily of the tyrosine kinase receptors (19). The *flk-1* gene was isolated from an embryonic liver-derived cell population enriched with primitive hematopoietic stem cells (20, 21) and encodes a tyrosine kinase receptor that is homologous to *flt*. The product of the *flk-1* gene and the product of its human homologue KDR also bind VEGF and undergo autophosphorylation in response to VEGF. However, it is not clear yet whether *flk-1* or *flt* can transduce a VEGF induced mitogenic signal (22, 23). Both *flt* and *flk-1* contain seven immunoglobulin-like loops in their extracellular domains, whereas other members of the PDGF receptor family such as *c-kit* or *c-fms* contain only five immunoglobulin-like loops (20).

We have expressed the *flk-1* cDNA and a chimeric gene containing the extracellular domain of *flk-1* and the tyrosine kinase domain encoded by the *c-fms* gene in NIH-3T3 cells, and we report that both the chimera and the native receptor encoded by the *flk-1* gene bind VEGF<sub>165</sub> with high affinity. We show that heparin concentrations that inhibit the binding of <sup>125</sup>I-VEGF<sub>165</sub> to the chimeric receptor, and the VEGF<sub>165</sub> induced autophosphorylation of the chimeric receptor, potentiate the binding of VEGF<sub>165</sub> to endogenous receptors of NIH-3T3 cells. We also present evidence indicating that the binding of

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<sup>1</sup> The abbreviations used are: VEGF, vascular endothelial growth factor; VEGF<sub>165</sub>, 165-amino acid form of vascular endothelial growth factor; aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; BSA, bovine serum albumin; CSF-1, colony stimulating factor 1; EGF, epidermal growth factor; PAGE, polyacrylamide gel electrophoresis; SEAP, secreted alkaline-phosphatase.

VEGF<sub>165</sub> to a soluble fusion protein containing the entire extracellular domain of the *flk-1* receptor is strongly enhanced by heparin, but not by chondroitin sulfate or desulfated heparin.

# EXPERIMENTAL PROCEDURES

**Materials**—Human recombinant VEGF<sub>165</sub> was purified from the conditioned medium of Sf-9 insect cells infected with a baculovirus-based expression vector for VEGF<sub>165</sub> as described (24). The factor was highly purified as determined by SDS-PAGE chromatography followed by silver staining, using three purification steps, including hydrophobic chromatography, cation-exchange chromatography, and heparin-Sepharose affinity chromatography. Recombinant human bFGF and aFGF were produced in bacteria as described (15). Recombinant PDGF-BB was kindly given by Dr. I. Vlodavsky (Hadassah-Hebrew University Hospital, Jerusalem). EGF was kindly given by Dr. Gospodarowicz (Chiron Inc.). The pMFG expression vector was kindly given by Dr. Richard Mulligan (Whitehead Institute for Biomedical research, Cambridge, MA). Intestinal mucosa-derived heparin, and chondroitin sulfate A and C, were purchased from Sigma. Oversulfated and desulfated heparin were kindly given by Dr. Svahn (Kabi-Pharmacia Therapeutics, Stockholm, Sweden). Rat liver-derived heparan-sulfate was kindly given by Dr. J. T. Gallagher (Christie Hospital, Manchester, United Kingdom). Suramin was obtained from FBA. Heparin-Sepharose was purchased from Pharmacia. Na<sup>125</sup>I was purchased from New England Nuclear. Tissue culture plasticware was obtained from Nunc. Tissue culture media, sera, and cell culture supplements were from Beth-Haemek Biological Industries. Prestained high molecular weight size markers were purchased from Bio-Rad. Disuccinimidyl suberate was obtained from Pierce Chemical Co. Anti-alkaline phosphatase antibodies (A-018-01 antibody) were from Medix Biotech. The anti-*c-fms* antibodies were produced against a C-terminal peptide (GDIAQLLPNNYQFC). CSF-1 was purchased from Genzyme. All of the other chemicals were purchased from Sigma.

**Construction of Expression Plasmids Encoding *flk-1*/SEAP and *flk-1*/*c-fms* Fusion Proteins**—An antisense oligonucleotide (5'-GCCA-GATCTTTCCCAAGTTGGCTCTTTC-3') fusing the last codon (underlined) of the *flk-1* extracellular domain (20) to a *Bgl*I site (bold) was used in conjunction with a sense *flk-1* oligonucleotide (position 1880 in the *flk-1* sequence) to amplify a small fusion fragment. Digestion of the fragment with *Bgl*I and *Eco*RI (position 2452 in the *flk-1* sequence) and gel purification yielded a junction fragment. A 5' *flk-1* fragment was prepared by sequential amplification and joining of the *flk-1* sequences between positions 120 and 1739 from two plasmids followed by the addition of *Hind*III linkers. This fragment was digested with *Hind*III and *Sal*I (position 535 after the ATG codon) and gel-purified. A fragment containing the bulk of the extracellular domain was purified following digestion with *Sal*I and *Eco*RI. All of the above fragments were simultaneously ligated with dephosphorylated *Hind*III- and *Bgl*I-digested Apta vector (25) and transformed into bacteria.

An oligonucleotide 5'-GGTGGCCAGCATCCCCCGATGAGTTC-CTC-3' joining *flk-1* sequences to *c-fms* at a position six amino acids upstream of the *c-fms* transmembrane domain (26) was synthesized for the construction of the *flk-1*/*c-fms* (the last *flk-1* amino acid is underlined), and its complement was used to amplify two fragments overlapping at the position of gene fusion. The oligonucleotides used were at positions 1880 of *flk-1* and 2032 of *c-fms*, respectively. Both of these fragments were mixed and re-amplified with the *flk-1* and *c-fms* oligonucleotides (1880 and 2032) to create the fusion fragment. The rest of the cDNA encoding the extracellular domain of *flk-1* was grafted to this fusion fragment as described for the creation of the *flk-1*/SEAP encoding sequence. The sequence encoding the intracellular and transmembrane domains of *c-fms* was grafted to this fusion fragment in a similar manner. The DNA encoding the entire chimera was then subcloned into the pMFG expression vector and expressed in NIH-3T3 cells.

**Purification of the *flk-1*/SEAP Fusion Protein**—Ten liters of conditioned medium from *flk-1*/SEAP transfected NIH-3T3 cells were concentrated 10-fold by tangential flow ultra-filtration on a 30-kDa cut-off membrane. Following concentration, the conditioned medium was clarified by filtration through a 0.2- $\mu$  filter and then loaded onto a 15-ml monoclonal anti-SEAP-Sepharose column equilibrated previously with 0.1 M Tris-HCl, pH 7.6, 0.5 M NaCl, and 2 mM EDTA. The sample was recirculated over the column five times and then washed with 10 bed volumes of 0.2 M glycine HCl, pH 2.8, 0.5 M NaCl, and fractions were immediately neutralized with 2 M Tris base. Fractions containing purified *flk-1*/SEAP were pooled, dialyzed into 0.01 M phosphate-buffered saline, pH 7.2, and frozen at -20 °C. The purity of the *flk-1*/SEAP

preparation was >90% as determined by SDS-PAGE and N terminus sequencing.

**Cell Culture**—NIH-3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine, and antibiotics. Human umbilical vein-derived endothelial cells were maintained, and their proliferation in response to various growth factors monitored, as described previously (3, 27).

**Binding and Cross-linking of <sup>125</sup>I-VEGF<sub>165</sub>**—Iodination of human recombinant VEGF<sub>165</sub> was performed using the chloramine-T method, as described previously (12). The specific activity of the <sup>125</sup>I-VEGF<sub>165</sub> was about 10<sup>6</sup> cpm/ng. To cross-link <sup>125</sup>I-VEGF<sub>165</sub> to the *flk-1*/SEAP fusion protein, *flk-1*/SEAP (100 ng/ml) and <sup>125</sup>I-VEGF<sub>165</sub> (10 ng/ml) were incubated at room temperature for 1 h in binding buffer containing 10 mM HEPES, 150 mM NaCl, and 20  $\mu$ g/ml bovine serum albumin (BSA). The cross-linker (disuccinimidyl suberate) was then added to a final concentration of 0.2 mM for 15 min at room temperature, and the reaction was stopped with 20 mM glycine. The binding and cross-linking of <sup>125</sup>I-VEGF<sub>165</sub> to *flk-1* and to *flk-1*/*c-fms* transfected and nontransfected NIH-3T3 cells were performed, and cross-linked complexes visualized, as described previously (12, 15).

**VEGF<sub>165</sub>-induced Autophosphorylation of the *flk-1*/*c-fms* Chimeric Receptor in Transfected NIH-3T3 Clone C4 Cells**—Confluent cells in a 10-cm dish were transferred to serum-free medium containing 0.05 mM sodium orthovanadate, and the cells were incubated 4 more h in this medium at 37 °C. Subsequently, the medium was changed to serum-free medium containing 0.1% BSA and growth factors were added to the desired concentrations for 1–10 minutes. Following stimulation the medium was aspirated, and the cells were washed quickly with ice-cold Dulbecco's phosphate-buffered saline containing 1 mM sodium orthovanadate. The cells were then lysed with ice-cold lysis buffer (10 mM Tris-HCl, pH 7.4, 1% Triton X-100, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 2 mM sodium orthovanadate, 5 mM ZnCl<sub>2</sub>, 5 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml aprotinin, and 0.1 mg/ml leupeptin). Lysates were transferred to chilled Eppendorf tubes and centrifuged 5 min at 12,000  $\times$  g at 4 °C. Supernatants from each lysate were incubated with 5  $\mu$ g of affinity-purified antibody directed against *c-fms* and protein A-Sepharose for 2 h at 4 °C with constant shaking. Beads were subsequently washed once with wash buffer A (10 mM Tris-HCl, pH 8, 0.2% Triton X-100, 150 mM NaCl, 2 mM EDTA, and 1 mM sodium orthovanadate), once more with the same buffer containing 0.5 M NaCl, followed by two washes with 10 mM Tris-HCl pH 8. The beads were boiled in 2  $\times$  SDS buffer and the supernatant, separated on a 4–12% gradient SDS-PAGE gel. Proteins were transferred to nitrocellulose by electroblotting. Phosphoproteins on the nitrocellulose were detected using an anti-phosphotyrosine antibody (Upstate Biotechnology, Inc.), and bound antibodies were visualized using the ECL system (Amersham).

# RESULTS

**The Receptor Encoded by the *flk-1* Gene Binds <sup>125</sup>I-VEGF<sub>165</sub> with High Affinity**—Full-length *flk-1* cDNA was subcloned into the molony murine leukemia virus long terminal repeat driven expression vector pMFG. This expression vector was stably co-transfected into both NIH-3T3 cells and baby hamster kidney-derived fibroblast (BHK-21 cells) (28). The presence of VEGF binding sites was examined in geneticin-resistant clones of cells using <sup>125</sup>I-VEGF<sub>165</sub> binding (12). The transfection resulted in the appearance of clones which expressed low densities (no more than 600 receptors/cell) of high affinity <sup>125</sup>I-VEGF<sub>165</sub> binding sites (not shown). We could not detect a mitogenic response to VEGF<sub>165</sub> in these cells nor could we detect VEGF<sub>165</sub>-induced autophosphorylation.

To try to overcome these problems we have expressed in NIH-3T3 cells a chimeric cDNA containing the extracellular domain of *flk-1* fused to the transmembrane and intracellular domains of the CSF-1 receptor (*c-fms*). Saturation binding experiments in which increasing concentrations of <sup>125</sup>I-VEGF<sub>165</sub> were bound to NIH-3T3 clone C4 cells expressing the chimeric *flk-1*/*c-fms* receptors were analyzed by the method of Scatchard using the ligand program (29). These experiments revealed one class of high affinity binding sites for <sup>125</sup>I-VEGF<sub>165</sub> with a dissociation constant of  $1.9 \times 10^{-11}$  M. The density of these *flk-1*/*c-fms* receptors was 2800 receptors/cell (Fig. 1B).

Cross-linking experiments revealed a <sup>125</sup>I-VEGF<sub>165</sub>-contain-

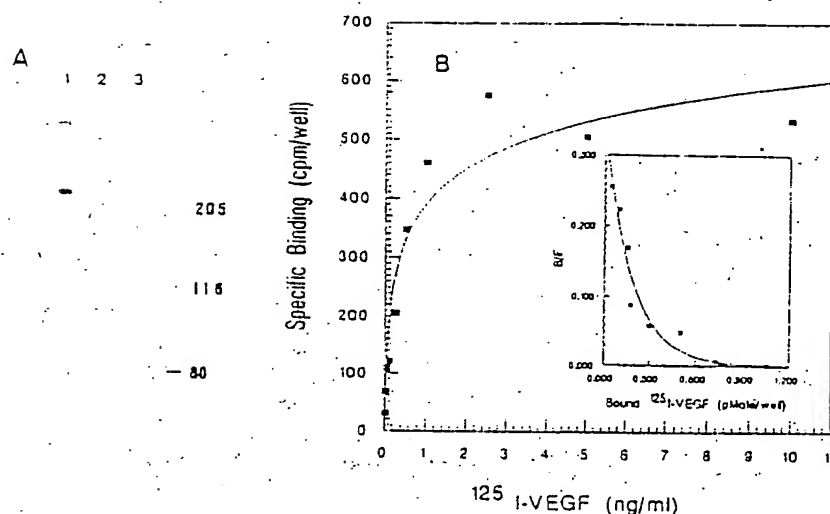


FIG. 1. Binding and cross-linking of  $^{125}\text{I}$ -VEGF<sub>165</sub> to *flk-1/c-fms*-producing cells. A, expression of the *flk-1/c-fms* chimeric receptor in NIH-3T3 cells. *Flk-1/c-fms*-producing (lanes 1 and 2) and nonproducing (lane 3) NIH-3T3 cells were grown to subconfluence in 6-cm dishes. The binding of  $^{125}\text{I}$ -VEGF<sub>165</sub> (5 ng/ml) to the cells in the presence (lane 2) or absence (lanes 1 and 3) of 0.5  $\mu\text{g}/\text{ml}$  of unlabeled VEGF<sub>165</sub>, and the subsequent cross-linking of bound  $^{125}\text{I}$ -VEGF<sub>165</sub> to the receptors were conducted as described under "Materials and Methods." B, representative saturation binding experiment with *flk-1/c-fms* expressing NIH-3T3 cells. Cells were grown to subconfluence in 24-multiwell dishes (160,000 cells/well). Increasing concentrations of  $^{125}\text{I}$ -VEGF, ranging from 10  $\text{pg}/\text{ml}$  to 10  $\text{ng}/\text{ml}$ , were bound to the cells for 2 h at 4  $^{\circ}\text{C}$ . Nonspecific binding was measured in the presence of 0.5  $\mu\text{g}/\text{ml}$  unlabeled VEGF, and the specific binding was calculated by the subtraction of the nonspecific binding from the total binding. At the end of the binding reaction, the cells were washed three times with 1 ml of ice-cold Dulbecco's phosphate-buffered saline containing 1  $\text{mg}/\text{ml}$  BSA. The cells were then solubilized with 0.5 ml of 0.2  $\text{N}$  NaOH. Aliquots were counted in a  $\gamma$ -counter. Shown is a saturation curve in which the amount of specifically bound  $^{125}\text{I}$ -VEGF<sub>165</sub> is plotted as a function of added  $^{125}\text{I}$ -VEGF<sub>165</sub> concentrations and a Scatchard plot derived from the saturation curve (inset).

ing complex of 210 kDa in NIH-3T3 clone C4 cells which is not present in the nontransfected NIH-3T3 cells (Fig. 1A, compare lanes 1 and 3). The formation of this complex was inhibited by an excess of unlabeled VEGF<sub>165</sub> (Fig. 1A, lane 2). The chimeric receptor was autophosphorylated in response to VEGF<sub>165</sub> in a dose-dependent fashion (Fig. 2). Because of the relatively high expression levels of the *flk-1/c-fms* chimeric receptor in NIH-3T3 clone C4 cells, and because VEGF<sub>165</sub>-induced signal transduction could be observed in these cells, we have chosen to use the NIH-3T3 clone C4 cells for further experiments.

**The Effect of Heparin on the Binding of  $^{125}\text{I}$ -VEGF<sub>165</sub> to the Endogenous VEGF Receptors and to *flk-1/c-fms* Chimeric Receptors Expressed in NIH-3T3 Cells**—In the absence of exogenous heparin the endogenous VEGF receptors of the NIH-3T3 cells (15) are barely detectable (Fig. 3, lane 1). However, when low concentrations of heparin (1  $\mu\text{g}/\text{ml}$ ) are included in the binding reaction, two  $^{125}\text{I}$ -VEGF<sub>165</sub>-receptor complexes can be detected (Fig. 3, lane 2). The binding of  $^{125}\text{I}$ -VEGF<sub>165</sub> to these receptors is inhibited when 0.5  $\mu\text{g}/\text{ml}$  of unlabeled VEGF<sub>165</sub> are added to the binding reaction (not shown). In contrast, the binding of  $^{125}\text{I}$ -VEGF<sub>165</sub> to the *flk-1/c-fms* chimeric receptors expressed in transfected NIH-3T3 clone C4 cells was readily detectable, even in the absence of added heparin (Fig. 1A and Fig. 3, lane 3). As expected, the endogenous receptors of the transfected cells were not seen when the binding was done in the absence of heparin (Fig. 1A and Fig. 3, lane 3). No significant effect of heparin on  $^{125}\text{I}$ -VEGF<sub>165</sub> binding to the *flk-1/c-fms* chimeric receptors was observed when heparin concentrations lower than 1  $\mu\text{g}/\text{ml}$  were included in the binding reaction (not shown). When 1  $\mu\text{g}/\text{ml}$  heparin was added to the binding reaction, the binding of  $^{125}\text{I}$ -VEGF<sub>165</sub> to the *flk-1/c-fms* chimeric receptor was significantly inhibited, whereas the binding of  $^{125}\text{I}$ -VEGF<sub>165</sub> to the endogenous VEGF receptors of the transfected cells was potentiated (Fig. 3, lane 4). Similar concentrations of heparin also inhibited partially the VEGF<sub>165</sub>-induced autophosphorylation of the transfected *flk-1/c-fms* chimeric re-

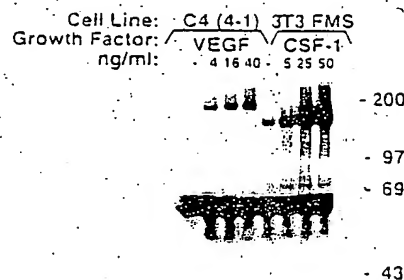


FIG. 2. VEGF<sub>165</sub> induces autophosphorylation of *flk-1/c-fms* chimeric receptors. NIH-3T3 clone C4 and NIH-3T3/*fms* cells were grown in 10-cm dishes and shifted to serum-free medium for 4 h before induction with growth factors as described under "Materials and Methods." The cells were stimulated for 8 min with the indicated concentrations of VEGF<sub>165</sub> or CSF-1. Following stimulation, the cells were lysed and the receptors immunoprecipitated with anti-*c-fms* antibodies as described under "Materials and Methods." Immunoprecipitated material was chromatographed on a SDS-PAGE gel, transferred to nitrocellulose, and tyrosine-phosphorylated proteins visualized as described under "Materials and Methods."

ceptors, indicating that the heparin-induced inhibition of VEGF<sub>165</sub> binding to the transfected receptors is accompanied by reduced receptor function (Fig. 4). A higher concentration of heparin (10  $\mu\text{g}/\text{ml}$ ) inhibited the binding of  $^{125}\text{I}$ -VEGF<sub>165</sub> to the endogenous and to the transfected VEGF receptors (Fig. 3, lane 5). It therefore follows that in the presence of 1  $\mu\text{g}/\text{ml}$  heparin the binding of  $^{125}\text{I}$ -VEGF<sub>165</sub> to one type of VEGF receptor is potentiated, whereas the binding of  $^{125}\text{I}$ -VEGF<sub>165</sub> to another class of VEGF receptors is inhibited, although both receptor types reside in the same cell.

**The Binding of  $^{125}\text{I}$ -VEGF<sub>165</sub> to a Soluble *flk-1*/SEAP Fusion Protein in a Cell-free Environment Is Potentiated by Heparin**—NIH-3T3 cells, as well as most other cell types, contain cell surface-associated heparin-like molecules (30). Therefore the modulatory effects of exogenously added heparin-like mol-

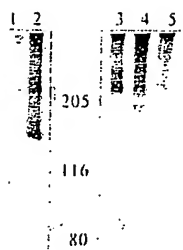


Fig. 3. The effect of heparin on the binding of  $^{125}$ I-VEGF<sub>165</sub> to the endogenous VEGF receptors and to the *flk-1/c-fms* chimeric receptors of NIH-3T3 clone C4 cells.  $^{125}$ I-VEGF<sub>165</sub> (20 ng/ml) was bound to parental NIH-3T3 cell (lanes 1 and 2), and a lower concentration (5 ng/ml) was bound to *flk-1/c-fms*-expressing NIH-3T3 clone C4 cells (lanes 3–5), in the absence (lanes 1 and 3) or in the presence of 1 µg/ml (lanes 2 and 4) or 10 µg/ml (lane 5) heparin. The subsequent cross-linking of bound  $^{125}$ I-VEGF<sub>165</sub> to the receptors, and visualization of cross-linked complexes were done as described under "Materials and Methods." Lanes 1 and 2 were autoradiographed for 9 days, and lanes 3–5 were autoradiographed for 5 days.

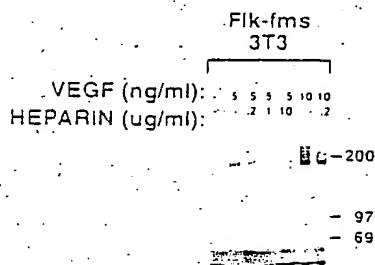


Fig. 4. Inhibition of VEGF<sub>165</sub>-induced phosphorylation of *flk-1/c-fms* by heparin. NIH-3T3 clone C4 cells were grown in 10-cm dishes and shifted to serum-free medium for 4 h before the experiment as described under "Materials and Methods." The cells were stimulated with VEGF<sub>165</sub> at 5 or 10 ng/ml for 8 min in the absence or presence of the indicated concentrations of heparin. Following stimulation, cells were lysed and the receptors immunoprecipitated with anti-*c-fms* antibodies as described under "Materials and Methods." Immunoprecipitated material was chromatographed on a SDS-PAGE gel, transferred to nitrocellulose, and tyrosine-phosphorylated proteins were visualized as described under "Materials and Methods."

ecules on  $^{125}$ I-VEGF<sub>165</sub> binding are superimposed on the effects of the endogenous cell surface-bound heparin-like molecules. To study the interaction between VEGF<sub>165</sub> and the *flk-1* receptor in a controlled environment in which the composition of glycosaminoglycans can be controlled precisely, we produced a soluble fusion protein consisting of the extracellular domain of *flk-1* fused to secreted human placental alkaline phosphatase (SEAP). The *flk-1*/SEAP fusion protein was not retained on a heparin-Sepharose column and specifically inhibited the VEGF<sub>165</sub>-induced, but not the basic fibroblast growth factor-induced, proliferation of human umbilical vein-derived endothelial cells by more than 70% (not shown).

To study the binding of  $^{125}$ I-VEGF<sub>165</sub> to the *flk-1*/SEAP fusion protein,  $^{125}$ I-VEGF<sub>165</sub> was incubated with the fusion protein and bound  $^{125}$ I-VEGF<sub>165</sub> was subsequently cross-linked to the soluble receptor. An excess of BSA was added to the binding reaction in order to inhibit nonspecific binding. Labeled  $^{125}$ I-VEGF<sub>165</sub>/*flk-1*/SEAP complexes of 205 kDa could be detected following cross-linking, but the efficiency of complex formation in the absence of heparin was low (Fig. 5A and Fig. 5B, lanes 1 and 3).  $^{125}$ I-VEGF<sub>165</sub>-receptor complexes could be seen under these conditions only after prolonged exposure (not shown). This heparin-independent binding was more prominent when high concentrations of  $^{125}$ I-VEGF<sub>165</sub> (40 ng/ml) were used (not shown). The addition of 0.1 µg/ml of heparin to the binding

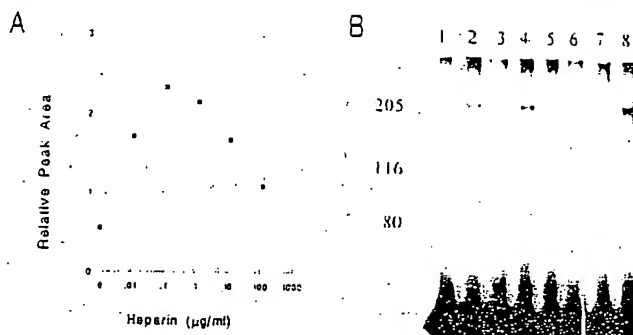


Fig. 5. The effect of various glycosaminoglycans on the binding of  $^{125}$ I-VEGF<sub>165</sub> to the *flk-1*/SEAP fusion protein. A, the effect of increasing concentrations of heparin on the binding of  $^{125}$ I-VEGF<sub>165</sub> to *flk-1*/SEAP.  $^{125}$ I-VEGF<sub>165</sub> (10 ng/ml) was bound to soluble *flk-1*/SEAP (100 ng/ml) in the presence of increasing concentrations of heparin as indicated. Binding was performed for 1 h at room temperature. The subsequent cross-linking of bound  $^{125}$ I-VEGF<sub>165</sub> to the *flk-1*/SEAP fusion protein and the visualization of cross-linked complexes were done as described under "Materials and Methods." The labeling density of the  $^{125}$ I-VEGF<sub>165</sub>/*flk-1*/SEAP complexes that were formed (see B) were determined using a Cliniscan-2 densitometer, and plotted as a function of heparin concentration. B, the effect of native and modified heparins and of various glycosaminoglycans on the binding of  $^{125}$ I-VEGF<sub>165</sub> to *flk-1*/SEAP.  $^{125}$ I-VEGF<sub>165</sub> (10 ng/ml) was bound to soluble *flk-1*/SEAP (100 ng/ml) in the presence of the following additions: lanes 1 and 3, no additions; lanes 2 and 4, 0.1 µg/ml heparin; lane 5, 0.1 µg/ml chondroitin sulfate A; lane 6, 0.1 µg/ml chondroitin sulfate C; lane 7, 0.1 µg/ml N/O-desulfated heparin; lane 8, 0.005 µg/ml O-sulfated heparin. Binding was performed for 1 h at room temperature. The subsequent cross-linking of bound  $^{125}$ I-VEGF<sub>165</sub> to the *flk-1*/SEAP fusion protein and the visualization of cross-linked complexes were done as described under "Materials and Methods."

reaction potentiated the binding of  $^{125}$ I-VEGF<sub>165</sub> to the soluble receptor (Fig. 5B; lanes 2 and 4). Densitometric analysis shows that 10 ng/ml of heparin already produce a 3-fold potentiation of  $^{125}$ I-VEGF<sub>165</sub> binding to the soluble receptor (Fig. 5A). A maximal 4-fold potentiation of  $^{125}$ I-VEGF<sub>165</sub> binding was produced in the presence of 0.1 µg/ml heparin, and heparin concentrations higher than 10 µg/ml progressively inhibited the binding compared with the maximal binding level observed in the presence of 0.1 µg/ml heparin (Fig. 5A). Labeled complexes were not formed when  $^{125}$ I-VEGF<sub>165</sub> monomers were used instead of dimers (12) or when the binding of  $^{125}$ I-VEGF<sub>165</sub> was performed using SEAP instead of the *flk-1*/SEAP fusion protein (not shown). The heparin concentrations that begin to produce inhibition of  $^{125}$ I-VEGF<sub>165</sub> binding to the *flk-1*/SEAP fusion protein are higher than the heparin concentrations that are required for partial inhibition of  $^{125}$ I-VEGF<sub>165</sub> binding to the *flk-1/c-fms* chimeric receptors of transfected NIH-3T3 cells (Fig. 3). Thus 1 µg/ml heparin still potentiates strongly  $^{125}$ I-VEGF<sub>165</sub> binding to *flk-1*/SEAP, but similar concentrations of heparin already inhibit the binding of  $^{125}$ I-VEGF<sub>165</sub> to the *flk-1/c-fms* chimera expressed in transfected NIH-3T3 cells (Fig. 3).

The potentiating effect of heparin appeared to be specific, since identical concentrations of chondroitin sulfate A or chondroitin sulfate C did not produce any enhancement of  $^{125}$ I-VEGF<sub>165</sub> binding (Fig. 5B, lanes 5 and 6, respectively). A similar concentration of rat liver-derived heparan sulfate produced a 2-fold potentiation of  $^{125}$ I-VEGF<sub>165</sub> binding to *flk-1*/SEAP, indicating that the potentiating effects was specific to heparin-like molecules. High concentrations of heparan sulfate (100 µg/ml or more) inhibited even the basal binding of  $^{125}$ I-VEGF<sub>165</sub> that is observed in the absence of added heparin (not shown). The overall sulfation level of heparin seemed to be critical for the potentiation of  $^{125}$ I-VEGF<sub>165</sub> binding to *flk-1*/SEAP. When N/O-desulfated heparin (0.1 µg/ml; total sulfate content, 1%) was



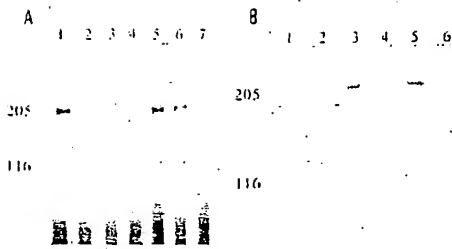


FIG. 6. Specificity of  $^{125}\text{I}$ -VEGF<sub>165</sub> binding to the soluble *flk-1*/SEAP receptor. A, the effect of various growth factors on the binding of  $^{125}\text{I}$ -VEGF<sub>165</sub> to *flk-1*/SEAP.  $^{125}\text{I}$ -VEGF<sub>165</sub> (10 ng/ml) was bound to the *flk-1*/SEAP protein (100 ng/ml) in the presence of 0.1  $\mu\text{g/ml}$  heparin and in the presence of the following additions: lane 1, no additions; lane 2, unlabeled VEGF<sub>165</sub> (0.5  $\mu\text{g/ml}$ ); lane 3, aFGF (0.5  $\mu\text{g/ml}$ ); lane 4, bFGF (0.5  $\mu\text{g/ml}$ ); lane 5, PDGF (0.5  $\mu\text{g/ml}$ ); lane 6, EGF (0.5  $\mu\text{g/ml}$ ); lane 7, suramin (1 mM). The binding, the subsequent cross-linking of bound  $^{125}\text{I}$ -VEGF<sub>165</sub> to the *flk-1*/SEAP fusion protein, and the visualization of cross-linked complexes were done as described under "Materials and Methods." B, modulation of the inhibitory effect of bFGF by heparin.  $^{125}\text{I}$ -VEGF<sub>165</sub> (10 ng/ml) was bound to the *flk-1*/SEAP protein (100 ng/ml) in the absence of heparin (lanes 1 and 2) or in the presence of 0.1  $\mu\text{g/ml}$  heparin (lanes 3 and 4) or 1  $\mu\text{g/ml}$  heparin (lanes 5 and 6). In addition, bFGF (0.5  $\mu\text{g/ml}$ ) was added to some of the binding reactions (lanes 2, 4, and 6). The binding, the subsequent cross-linking of bound  $^{125}\text{I}$ -VEGF<sub>165</sub> to the *flk-1*/SEAP fusion protein, and the visualization of cross-linked complexes were done as described under "Materials and Methods."

added instead of heparin, no enhancement of  $^{125}\text{I}$ -VEGF<sub>165</sub> binding could be observed (Fig. 5, lane 7). When *O*-oversulfated heparin was used (5 ng/ml; total sulfate content, 16%) (31), the enhancement of  $^{125}\text{I}$ -VEGF<sub>165</sub> binding appeared to be more potent than the enhancement achieved with native heparin and was maximal at 5 ng/ml of oversulfated heparin (compare Fig. 5, lanes 3 and 4). A higher concentration of *O*-oversulfated heparin (0.1  $\mu\text{g/ml}$ ) completely inhibited the binding of  $^{125}\text{I}$ -VEGF<sub>165</sub> to *flk-1*/SEAP, whereas native heparin caused a similar inhibition of binding only when 100  $\mu\text{g/ml}$  were included in the binding reaction (not shown).

**The Specificity of the Interaction of the Soluble *flk-1*/SEAP with VEGF<sub>165</sub>**—To examine the specificity of the interaction between  $^{125}\text{I}$ -VEGF<sub>165</sub> and the soluble *flk-1*/SEAP receptor, binding and cross-linking experiments were conducted in the presence of 0.1  $\mu\text{g/ml}$  heparin and various other substances. The binding of  $^{125}\text{I}$ -VEGF<sub>165</sub> to the soluble *flk-1*/SEAP receptor was completely inhibited by 0.5  $\mu\text{g/ml}$  of unlabeled VEGF<sub>165</sub> (Fig. 6A, lane 2) and by suramin (Fig. 6A, lane 7), a known inhibitor of angiogenesis (32) that inhibits the binding of VEGF<sub>165</sub> to its receptors on vascular endothelial cells (12). The binding of  $^{125}\text{I}$ -VEGF<sub>165</sub> was not inhibited by 0.5  $\mu\text{g/ml}$  of PDGF or by 0.5  $\mu\text{g/ml}$  EGF (Fig. 6A, lanes 5 and 6). In contrast, aFGF (0.5  $\mu\text{g/ml}$ ) (Fig. 6A, lane 3) and bFGF (0.5  $\mu\text{g/ml}$ ) (Fig. 6A, lane 4 and Fig. 6B, lane 4) inhibited the binding. Because these two factors bind heparin with high affinity, we reasoned that they could perhaps bind free heparin during the binding reaction (33) and therefore make heparin unavailable to  $^{125}\text{I}$ -VEGF<sub>165</sub>, resulting in a partial inhibition of  $^{125}\text{I}$ -VEGF<sub>165</sub> binding. This assumption was supported by an experiment in which the binding to the *flk-1*/SEAP fusion protein was done in the presence of increasing concentrations of heparin and 0.5  $\mu\text{g/ml}$  bFGF (Fig. 6B). The inhibitory effect of bFGF was abolished when the binding of  $^{125}\text{I}$ -VEGF<sub>165</sub> to *flk-1*/SEAP was done in the presence of 1  $\mu\text{g/ml}$  heparin (Fig. 6B, lane 6). This experiment indicates that a potent growth factor like bFGF may, under appropriate conditions, serve in the role of a growth inhibitor using a mechanism involving competition for shared cell surface modulators of receptor binding such as heparin-like molecules.

## DISCUSSION

The *flk-1* gene encodes a tyrosine kinase receptor and was isolated from a cDNA library prepared from mouse fetal liver-derived cells enriched with primitive hematopoietic stem cells (20, 21, 34). The expression of the full-length *flk-1* cDNA in cells, or the expression of a cDNA encoding a chimeric receptor containing the extracellular domain of *flk-1* fused to the transmembrane and intracellular domains of the CSF-1 receptor (*c-fms*), results in the expression of high affinity binding sites for  $^{125}\text{I}$ -VEGF<sub>165</sub>. The affinity of  $^{125}\text{I}$ -VEGF<sub>165</sub> for these receptors was similar to the affinity of VEGF<sub>165</sub> to VEGF receptors of vascular endothelial cells. Because the chimeric receptor was expressed at higher levels in transfected cells, and because we could show VEGF<sub>165</sub>-induced autophosphorylation of the chimeric receptors, we have used cells expressing the chimeric receptor for further studies.

The binding of  $^{125}\text{I}$ -VEGF<sub>165</sub> to the endogenous VEGF receptors of vascular endothelial cells and to the small number of endogenous VEGF receptors found in NIH-3T3 cells is potentiated by the addition of 1  $\mu\text{g/ml}$  heparin (15). Unexpectedly, both the VEGF<sub>165</sub>-induced autophosphorylation of the chimeric *flk-1/c-fms* receptors expressed in the NIH-3T3 clone C4 cells and the binding of  $^{125}\text{I}$ -VEGF<sub>165</sub> to these receptors were inhibited by heparin concentrations equal or larger than 1  $\mu\text{g/ml}$ , whereas lower heparin concentrations had no effect. It follows that heparin can potentiate the binding of  $^{125}\text{I}$ -VEGF<sub>165</sub> to one class of VEGF receptors and inhibit the binding of  $^{125}\text{I}$ -VEGF<sub>165</sub> to another class of VEGF receptors simultaneously. These experiments indicate therefore that the effect that heparin will have on the binding of  $^{125}\text{I}$ -VEGF<sub>165</sub> to specific VEGF receptors depends not only on the heparin binding ability of the growth factor, but to some extent also on specific characteristics associated with specific VEGF receptor types.

It was recently reported that the ligand binding ability of fibroblast growth factor receptor-1 is regulated by the direct binding of heparin to the receptor (35). The effect of heparin on  $^{125}\text{I}$ -VEGF<sub>165</sub> binding could also be in part the result of a direct interaction between heparin and a subset of VEGF receptors. Alternatively, it is possible that heparin modulates the binding of VEGF<sub>165</sub> to its receptors indirectly through specific cell surface heparin-binding proteins. It was reported that vascular endothelial cells express cell surface heparin receptors (36), and it was shown that cell surface-bound heparin can potentiate the binding of  $^{125}\text{I}$ -VEGF<sub>165</sub> to the VEGF receptors of vascular endothelial cells (15). Taken together, the experiments suggest that the effect of heparin on the interaction of VEGF<sub>165</sub> with cell surface VEGF receptors is a complex process that needs to be studied using an experimental setup that will allow precise control of the binding environment.

We have taken a step toward the establishment of such an experimental setup by producing a soluble chimeric VEGF receptor containing the entire extracellular domain of *flk-1* fused to soluble alkaline phosphatase (25). This *flk-1*/SEAP fusion protein turned out to be a specific inhibitor of VEGF<sub>165</sub>-induced cell proliferation. This soluble receptor could perhaps be used in the future as an *in vivo* VEGF<sub>165</sub> antagonist. The availability of the fusion protein allowed us to conduct binding experiments in a precisely regulated cell free environment. The *flk-1*/SEAP-soluble receptor did not bind  $^{125}\text{I}$ -VEGF<sub>165</sub> efficiently in the absence of heparin, but addition of low heparin concentrations (as low as 5 ng/ml) to the binding reaction strongly potentiated the binding of  $^{125}\text{I}$ -VEGF<sub>165</sub> to *flk-1*/SEAP. The effect of heparin on  $^{125}\text{I}$ -VEGF<sub>165</sub> binding could not be mimicked by chondroitin sulfate, but heparan sulfate had a similar effect. The sulfation level of the heparin was important for the potentiating effect, since desulfated heparin had no activity,



whereas oversulfated heparin potentiated the binding of <sup>125</sup>I-VEGF<sub>165</sub> more efficiently than heparin. These experiments, done in a controlled cell free environment, indicate that heparin can directly modulate the binding of <sup>125</sup>I-VEGF<sub>165</sub> to *flk-1*/SEAP and that the modulation in this case is probably mediated exclusively through the interaction of heparin with <sup>125</sup>I-VEGF<sub>165</sub>, since *flk-1*/SEAP does not appear to bind to heparin-Sepharose.

In the presence of 1 µg/ml heparin, the binding of <sup>125</sup>I-VEGF<sub>165</sub> to the *flk-1*/c-fms receptors on transfected NIH-3T3 cells is inhibited, whereas the same concentration of heparin potentiates the binding of <sup>125</sup>I-VEGF<sub>165</sub> to the soluble *flk-1*/SEAP receptor. Since cell surfaces already contain heparin-like molecules, it follows that the effect of exogenous heparin on the binding of <sup>125</sup>I-VEGF<sub>165</sub> to a cell surface located VEGF receptor will be superimposed upon the effect of pre-existing cell surface associated heparin-like molecules. In addition, putative heparin binding cell surface receptors may also modulate the binding, resulting in complex final effects which are avoided by the usage of the soluble *flk-1*/SEAP receptors.

The binding of <sup>125</sup>I-VEGF<sub>165</sub> to the *flk-1*/SEAP-soluble receptor could not be inhibited by the growth factors PDGF and EGF (6–8). In contrast, high concentrations of bFGF and aFGF inhibited the binding of <sup>125</sup>I-VEGF<sub>165</sub> to the soluble *flk-1*/SEAP fusion protein. We found that these growth factors lost their ability to inhibit the binding of <sup>125</sup>I-VEGF<sub>165</sub> to the soluble *flk-1*/SEAP fusion protein when the binding experiments were conducted in the presence of high concentrations of heparin. It therefore follows that competition for available cell surface heparin-like molecules could perhaps function as an indirect cross-talk mechanism by which a growth factor such as bFGF may modulate the activity of another heparin binding growth factor such as VEGF<sub>165</sub>.

To conclude, our results indicate that heparin affects the interaction of VEGF<sub>165</sub> with various VEGF receptors by more than one mechanism. We have also shown that heparin can modulate the interaction of VEGF<sub>165</sub> with the VEGF binding domain of the *flk-1* receptor, even when the binding is done in an environment that contains only *flk-1*/SEAP receptors, VEGF, and heparin. The results of this study indicate that VEGF may play an important role in the maturation process of hematopoietic cells, since the *flk-1* cDNA was originally isolated from a cell population enriched with primitive hematopoietic stem cells (20).

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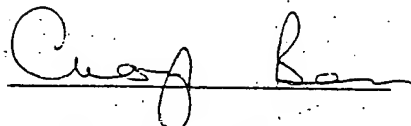
IN THE MATTER OF Australian Patent  
Application Serial No. 696764 by Human  
Genome Sciences, Inc.

-and-

IN THE MATTER OF Opposition thereto by  
Ludwig Institute for Cancer Research

THIS IS Exhibit PAWR-8  
referred to in the Statutory Declaration  
of Peter Adrian Walton Rogers  
made before me

DATED this 12<sup>th</sup> Day of November, 2001

A handwritten signature in cursive script, appearing to read 'Craig R. ...', written over a horizontal line.

(Signature of Witness)

Medical Practitioner

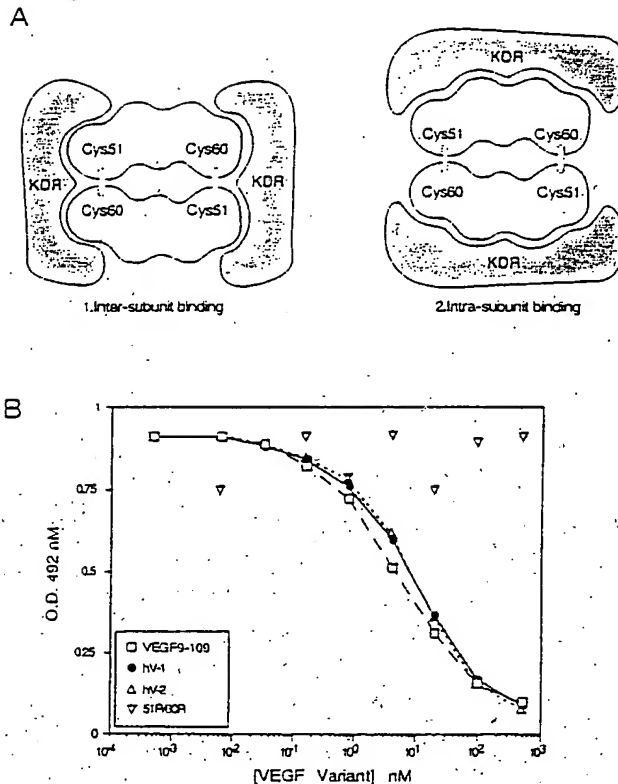


FIG. 2. Panel A, inter-subunit and intra-subunit models for binding two molecules of KDR receptor to dimeric VEGF. The inter-subunit disulfide bonds in VEGF are indicated. Panel B, displacement of biotinylated VEGF<sub>1-109</sub> from binding to monomeric KDR 1-7 by a monomeric form of VEGF<sub>1-109</sub> containing the double mutant C51R/C60R, a heterodimeric form of VEGF<sub>1-109</sub> that possesses either both binding sites (hV-2), or a single site at one pole of the hormone (hV-1) and the wild-type VEGF<sub>1-109</sub> with  $IC_{50}$  values  $\gg 500$ , 10, 8.9, and 4.6 nM, respectively. Dilutions of these VEGF variants were added with fixed amounts of biotinylated VEGF (1 nM) to KDR (0.5 nM) and incubated for 18 h. The complex was captured with a mAb to KDR (MAKD5) as described under "Experimental Procedures."

**The IgG-like Domains 2-3 in KDR Are Sufficient for High Affinity Binding**—To determine the minimal IgG-like domain requirements for binding of KDR to VEGF<sub>1-109</sub>, a series of deletions were produced in which each of the seven IgG-like domains were deleted from the carboxyl terminus of the extracellular domain. The deletion variants were expressed initially as dimeric proteins by fusion to the CH2-CH3 domain of an antibody (KDR-IgG). This was done to facilitate purification on a Protein A affinity column (12) and to compare their affinities to monomeric forms of KDR.

The choice of deletion junction was based on homology to other members of the IgG superfamily (16, 17): Systematic carboxyl-terminal domain deletions had virtually no effect on affinity for VEGF until IgG-like domain 3 was deleted (Table I); KDR 1-2 had an affinity that was  $>1000$ -fold reduced relative to KDR 1-3 but did show specific binding at concentrations above  $2 \mu\text{M}$  (data not shown). A variant of KDR missing the first NH<sub>2</sub>-terminal domain, KDR 2-3, bound nearly as well as the full-length KDR (Table I). These data suggest that domains 2-3 are most important for high affinity binding.

To determine if these deletions had caused misfolding of the molecules, we analyzed their binding to three different anti-KDR monoclonal antibodies (Table I), one of which (MAKD6)

TABLE I  
Analysis of deletions of the seven IgG-like domains in the extracellular domain of KDR

	$K_d$ (pM), <sup>a</sup> VEGF <sub>1-109</sub>	$EC_{50}$ (nM)		
		Non-blocking		Blocking
		MAKD1	MAKD5	MAKD6
KDR 1-7-IgG	51 ( $\pm 11$ )	2.11	1.51	0.45
KDR 1-5-IgG	143 ( $\pm 24$ )	2.13	1.05	0.41
KDR 1-4-IgG	57 ( $\pm 12$ )	1.74	0.95	0.44
KDR 1-3-IgG	101 ( $\pm 18$ )	1.86	1.12	0.51
KDR 1-2-IgG	$>100,000$	2.04	1.02	0.60
KDR 2-3-IgG	100	ND <sup>b</sup>	ND <sup>b</sup>	0.50
KDR1-7 monomer	4990 ( $\pm 700$ )	1.89	1.59	0.64
KDR1-3 monomer	3350 ( $\pm 1000$ )	1.81	0.88	0.53

<sup>a</sup> The binding affinity of KDR variants to VEGF<sub>1-109</sub> was measured using a competitive radioimmunoassay and <sup>125</sup>I-VEGF<sub>1-109</sub> (NEN Life Science Products Inc., DuPont) as a tracer except that the affinity for KDR 2-3-IgG was measured using biotinylated VEGF as tracer as described under "Experimental Procedures."

<sup>b</sup> ND, not detectable.

blocks binding of VEGF. The antibodies bound to nearly all the deletion variants with affinities virtually identical to the full-length KDR 1-7 ( $EC_{50} \sim 1$  nM). Deletion of domain 1 caused complete disruption for binding of the non-neutralizing antibodies (MAKD1 and MAKD5) but not the neutralizing antibody (MAKD6). Thus, the deletions do not grossly disrupt the structure of the molecules and locate the epitopes for MAKD1 and MAKD5 to domain 1 and for MAKD6 to domain 2. The fact that the antibody MAKD6, which blocks binding of VEGF, binds to domain 2 further supports the importance of domain 2 for binding VEGF.

To facilitate preparation of monomeric forms of KDR, a Gen-ense 1 protease cleavage site (18) was engineered at the junction of the last KDR IgG domain and the CH2 domain (19). The cleaved KDR was shown to be monomeric based on its mobility in nonreducing SDS-PAGE and gel filtration. Both the KDR 1-7 and KDR 1-3 monomers bound all three mAbs and equally well to VEGF (Table I). These results show the first three IgG-like domains are sufficient for binding of VEGF whether in monomeric or dimeric forms.

**One VEGF Dimer Binds Two Molecules of the Extracellular Domain of KDR**—To determine the stoichiometry of binding of VEGF<sub>1-109</sub> to the extracellular domain of KDR, we systematically varied the ratio of VEGF to KDR and determined the apparent size of the complexes by gel filtration. The glycosylated monomeric KDR 1-7 migrated as a single peak by gel filtration chromatography with an apparent molecular mass of  $\sim 250$  kDa (Fig. 3A). By comparison, the dimeric KDR 1-7-IgG migrated as a 600-kDa peak (data not shown). Upon addition of one equivalent of VEGF (dimer) per two equivalents of KDR (monomer), a single complex peak was formed of apparent molecular mass  $\sim 400$  kDa. A minor shoulder was seen that might represent the slight excess KDR monomer in the mixture. The fact that the 2:1 complex is smaller by gel filtration than expected from the sum of the component molecular masses (520 kDa) may be that VEGF aligns the receptor subunits in a more compact fashion.

Further additions of 2 and 3 equivalents of VEGF did not change the position of the high molecular weight peak, and excess VEGF accumulated as the free dimeric hormone (Fig. 3A). The height of the free VEGF peak was small because VEGF contains no tryptophan residues and therefore has a small molar absorbance at 280 nm. When the ratio of KDR to VEGF exceeded 2:1, free KDR 1-7 accumulated as a shoulder. The hV-1 heterodimeric variant of VEGF forms a 1:1 complex with monomeric KDR. This complex migrated at a position that was intermediate between the free KDR 1-7 and the 2:1

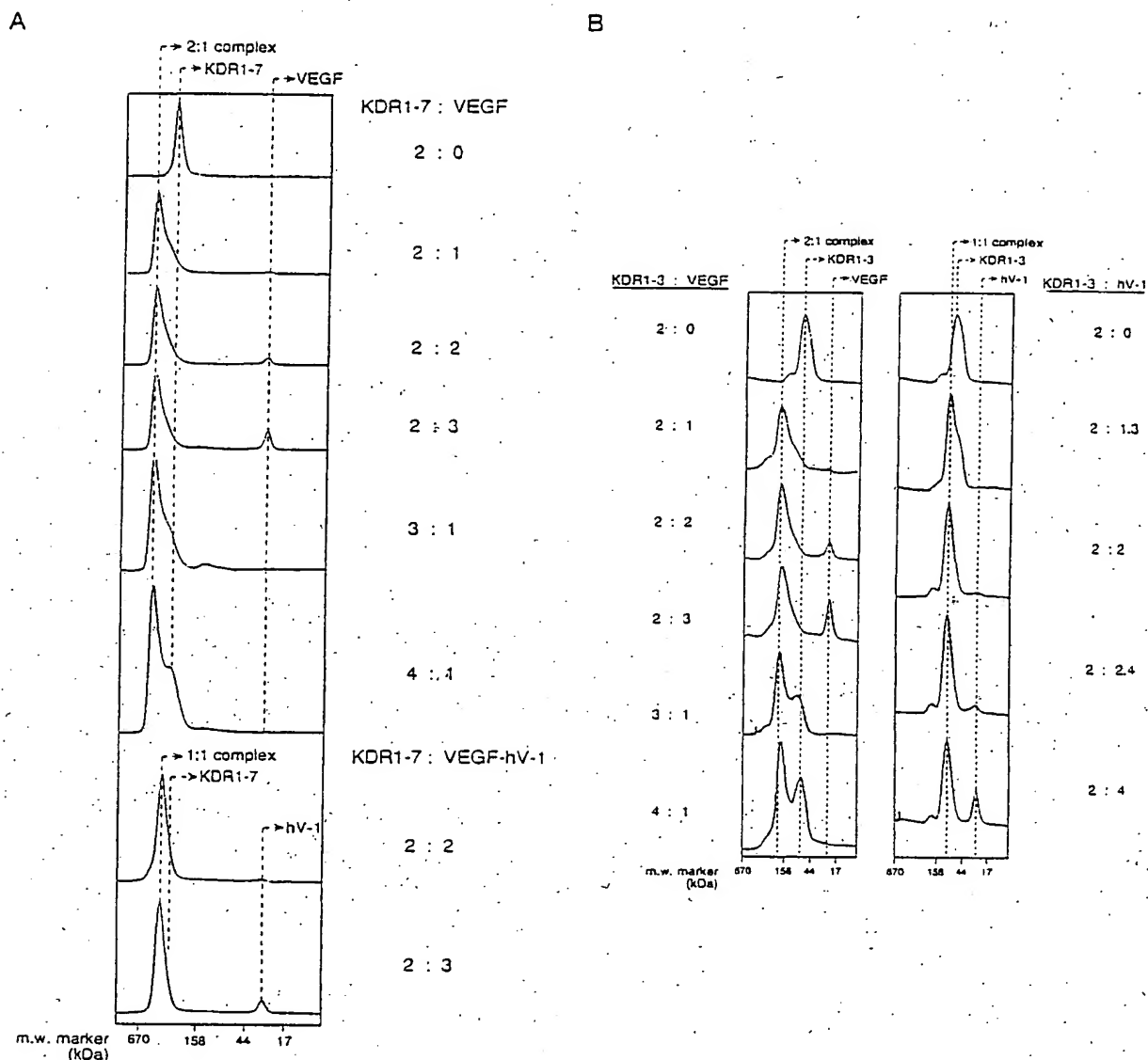


FIG. 3. Panel A, gel filtration chromatography of various ratios of KDR 1-7 monomer and VEGF dimer (upper six chromatograms) or VEGF heterodimer (hV-1) containing a single functional binding site (lower two chromatograms). The concentration of KDR 1-7 monomer was  $1 \mu\text{M}$  except at ratios of 3:1 and 4:1, where the concentration of KDR 1-7 was  $1.5$  and  $2 \mu\text{M}$ , respectively. The quantitation of protein was determined by amino acids hydrolysis and absorbance at  $280 \text{ nm}$ . Panel B, gel filtration chromatography of various ratios of KDR 1-3 monomer and VEGF dimer (six chromatograms on the left) or hV-1 (five chromatograms on the right). The concentration of KDR 1-3 monomer was held constant at  $1 \mu\text{M}$  except at ratios of 3:1 and 4:1, where KDR 1-3 monomer was  $1.5$  and  $2 \mu\text{M}$ , respectively.

KDR-VEGF complex. When the ratio of the hV-1 to KDR 1-7 exceeded unity, the free heterodimer accumulated in the chromatogram.

Parallel experiments were carried out with the monomeric form of KDR 1-3 (Fig. 3B). When no VEGF was present, KDR 1-3 migrated as a single peak of apparent molecular mass of  $\sim 70 \text{ kDa}$ . Addition of 1 equivalent of VEGF dimer to 2 equivalent of KDR 1-3 resulted in forming a peak with apparent molecular mass of  $\sim 160 \text{ kDa}$ . Addition of 2 and 3 equivalents of VEGF did not change the position of the complex peak, but free VEGF accumulated. Increasing additions of KDR 1-3 in excess of the 2:1 ratio to VEGF dimer showed increasing appearance of free KDR 1-3. A similar set of experiments with the hV-1 showed it maximally formed a 1:1 complex (Fig. 3B); when the

ratio of either the variant or KDR 1-3 was skewed from unity, the free excess component accumulated. These experiments explicitly show that two molecules of KDR bind to one VEGF dimer, and that form of KDR lacking IgG-like domains 4-7 are capable of producing the 2:1 complex in solution. When VEGF is engineered to have only one functional binding site (hV-1) it cannot dimerize the receptor *in vitro*.

**VEGF Binds Avidly to Dimeric versus Monomeric Forms of KDR**—Given the fact that the VEGF dimer binds two molecules of receptor we wished to determine to what extent pre-dimerization of the receptor influenced affinity. This can be readily seen by comparing the binding constants for the monomeric and dimeric forms of KDR (Table I). KDR-IgG fusions containing domains 1-7 or 1-3 bound 50-100-fold stronger

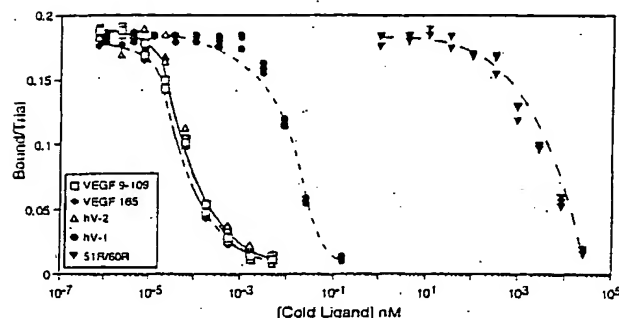


FIG. 4. VEGF containing two sites binds much stronger to predimerized forms of KDR. Displacement of  $^{125}\text{I}$ -VEGF $_{1-166}$  from the dimeric KDR 1-7-IgG is shown for wild-type VEGF $_{1-166}$ , VEGF $_{9-109}$ , the single disulfide heterodimer containing both binding sites (hV-2) or single binding site (hV-1), and monomeric VEGF $_{1-109}$ , C51R/C60R. The  $\text{IC}_{50}$  values from here and Fig. 2B are summarized in Table II.

than their monomeric counterparts. The affinity of the single-site heterodimeric VEGF for binding to the dimeric KDR IgG fusion was about 200-fold weaker than wild-type VEGF (Fig. 4). In contrast, binding to monomeric KDR for the heterodimer was only 2-fold weaker than native VEGF (Fig. 2B). These data, summarized in Table II, show that binding of dimeric VEGF to predimerized KDR is  $\sim 100$ -fold stronger than when either the hormone or receptor contains a single binding site.

**VEGF Binds Virtually the Same Way to Monomeric and Dimeric KDR**—Given the strong avidity component to binding of VEGF to its receptor, we wished to determine if VEGF binds the same way to monomeric and dimeric forms of KDR 1-7. We have previously reported the alanine scan of VEGF for binding to KDR-IgG (9). Here we analyze the binding of these same alanine mutants to monomeric KDR (Table III). The data show that the same set of alanine mutants that are most disruptive for binding to KDR-IgG are also strongly disruptive to binding monomeric KDR.

There are some subtle and systematic differences in the way the alanine mutants bind the monomeric *versus* dimeric KDR. For example M18A, I43A, I46A, E64A, and I83A were more disruptive to affinity (by factors ranging from 2–8-fold) when tested against the monomeric *versus* dimeric KDR. Only F17A was more disruptive to the dimer than the monomer (by  $\sim 2$ -fold). The biased suppression of the disruptive effects of the alanine mutations when binding to the dimer is likely caused from avidity in binding. We conclude there are no gross differences in the way monomeric and dimeric forms of KDR bind to VEGF.

**KDR Domains 1-3 Are Sufficient for Signaling in Cells**—NIH 3T3 fibroblast cells that contain the extracellular domain of colony-stimulating factor receptor fused to the transmembrane and intracellular domain of the Flt-4 receptor incorporate  $^3\text{H}$ thymidine and proliferate when treated with the colony-stimulating factor (20). To produce a VEGF responsive cell line, we made a similar chimera in which the seven IgG-like domains of KDR were linked to the transmembrane and intracellular kinase domain of Flt-4. At low concentrations of VEGF this cell line incorporated  $^3\text{H}$ thymidine with an  $\text{EC}_{50}$  of  $\sim 100$  pM (Fig. 5A); high concentrations of VEGF ( $> 1 \mu\text{M}$ ) showed inhibition. Such a bell-shaped dose-response curve is anticipated for a two-site hormone dimerizing two identical receptors (21). The hV-1 was inactive (Fig. 5A).

A similar construct was produced in which only domains 1-3 of KDR were linked to Flt-4. These cells also incorporated  $^3\text{H}$ thymidine in response to VEGF (Fig. 5B) but did so with a higher  $\text{EC}_{50}$  ( $\sim 10$  nM) and lower maximal response. We did not

TABLE II  
Summary of dissociation constants ( $K_d$ ) for binding of monomeric and dimeric forms of VEGF and KDR 1-7

VEGF $_{1-109}$	KDR	
	Monomer	Dimer
Monomer <sup>a</sup>	$\sim 20 \mu\text{M}$	$1.5 \mu\text{M}$
Single-site heterodimer (hV-1) <sup>a</sup>	$10 \text{ nM}$	$7 \text{ nM}$
Wild-type dimer <sup>b</sup>	$5 \text{ nM}$	$50 \text{ pM}$

<sup>a</sup> Data from Figs. 2B and 4. Data for the monomeric VEGF binding to monomeric KDR was from other experiments not shown.

<sup>b</sup> Data from Table I.

TABLE III  
The comparison of the effect alanine mutants of VEGF $_{1-109}$  on binding to KDR 1-7-IgG or KDR1-7 monomer

The relative binding affinity was expressed as the fold difference of alanine mutants with VEGF $_{1-109}$  in the competitive binding assay as described under "Experimental Procedures." Standard deviations in these measurements averaged  $\pm 25\%$  of the value shown. Residues are shown in two groups (e.g., F17A or I43A') to indicate that they are presented in the same epitope but from different subunits. Each mutant is present twice in the dimer.

	KDR-IgG dimer	KDR monomer
VEGF wt	1	1
F17A	230	91
M18A	11	98
E64A	120	460
I43A'	25	120
Y45A'	140	170
I46A'	400	1000
Q79A'	100	
I83A'	360	810

go to high enough concentrations to see inhibition by VEGF. The hV-1 was virtually inactive. Primary human umbilical vein endothelial cells (HuVEC) showed a bell-shaped dose-response curve (Fig. 5C). We resist making quantitative comparisons between HuVEC and KDR expressed NIH 3T3 cells given the fact that the HuVEC contain both KDR and Flt-1 receptors (22).

The difference in  $\text{EC}_{50}$  values and maximal response for the KDR 1-7 and KDR 1-3 cell lines likely resulted from the fact that the number of functional receptors on the KDR 1-3 cell line was at least 10-fold lower based upon binding of  $^{125}\text{I}$ -VEGF (data not shown). To explore the effect of receptor number on signaling directly we isolated three different clones of cells that varied over a range of 12-fold in the amount of the KDR 1-7 that specifically bound  $^{125}\text{I}$ -VEGF (Fig. 6A). The maximal levels of  $^3\text{H}$ thymidine incorporation correlated with the number of receptors expressed on these cells and the  $\text{EC}_{50}$  values correlated inversely with the number of receptors (Fig. 6B). It is interesting that the basal levels of  $^3\text{H}$ thymidine incorporation correlated with the receptor number as well, suggesting that receptors can preassociate and signal weakly in the absence of exogenous VEGF. All of the transfectants containing the KDR 1-3 construct expressed much lower levels of receptors which may suggest that domains 4-7 are important for high level expression and display of the receptor.

**Antagonism of VEGF Receptors by the Single-site Heterodimer of VEGF**—Given the ability of hV-1 to bind but not dimerize and activate KDR (Fig. 5), we studied its ability to antagonize signaling of KDR. Indeed the heterodimer antagonizes  $^3\text{H}$ thymidine incorporation in the 3T3 cells transfected with the chimeric KDR (1-7)-Flt-4 receptor and HuVEC with an  $\text{IC}_{50}$  of  $\sim 300$  and  $\sim 20$  nM, respectively (Fig. 7). The fact that the heterodimer is less effective on the 3T3-transfected cells *versus* HuVEC likely reflects the fact that the former expresses much higher levels of receptors (Fig. 6A).

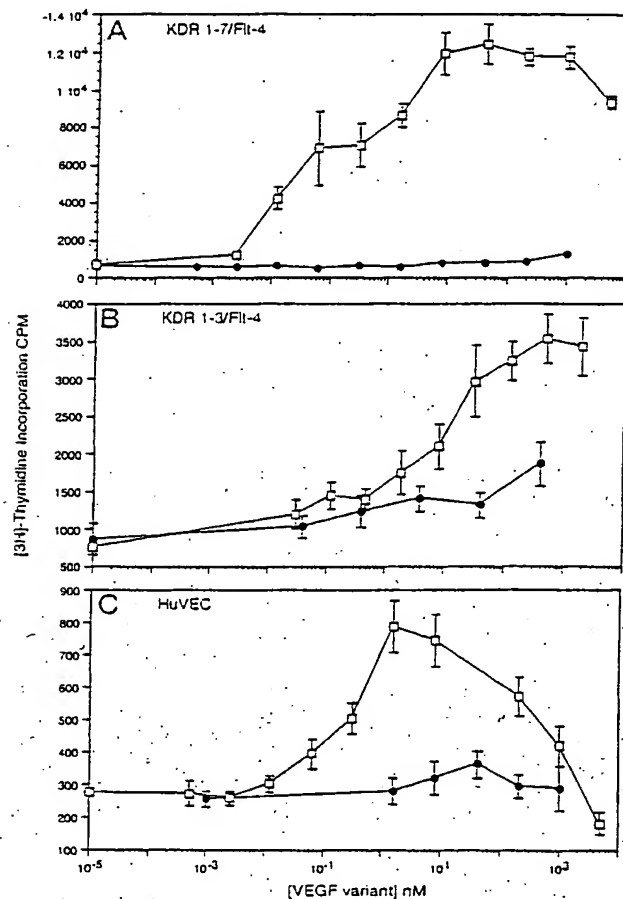


FIG. 5. The agonistic effects of VEGF variants on DNA synthesis of 3T3 cells stably expressing KDR 1-7 (ECD)/Flt4 (ICD) (Panel A), KDR 1-3 (ECD)/Flt4 (ICD) (Panel B), or HuVEC (Panel C). Cells were treated for 18 h with either VEGF<sub>9-109</sub> (open squares) or the VEGF<sub>1-109</sub> heterodimer with single binding site, hV-1 (closed circles). Cells were pulsed with [<sup>3</sup>H]thymidine for 6 h and analyzed as described under "Experimental Procedures."

#### DISCUSSION

**KDR Binds Across the VEGF Dimer Interface**—The data here combined with previous mutational analysis (9) suggest that binding occurs across the VEGF dimer interface (Fig. 2A). It may be a general feature of the cystine-knot hormones that the receptor binds at the interface between hormone subunits. A structure of domain 2 of the Flt-1 receptor bound to VEGF shows it binds across the dimer interface (23). A heterodimer containing one molecule of VEGF linked to its homolog, PLGF, is only 20–50-fold reduced as a mitogen on HuVEC cells (EC<sub>50</sub> ~ 50 nM) whereas the PLGF homodimers are inactive (24). The fact the VEGF/PLGF heterodimer shows any activity can be rationalized from our mutational analysis (Table III). Some of the critical binding determinants (Phe-17, Glu-64, Gln-79, and Ile-83) are conserved in PLGF and others are reasonably conservative substitutions (M18Q, I43V, Y45H and I46M). These later substitutions would likely have a much more dramatic effect when present in both subunits, thus accounting for the absence of significant mitogenic activity for the PLGF homodimer up to the concentrations that were tested (~1 μM). Similar observations have been made for homodimers and heterodimers of PDGF (isoforms AA, AB, and BB) for binding the

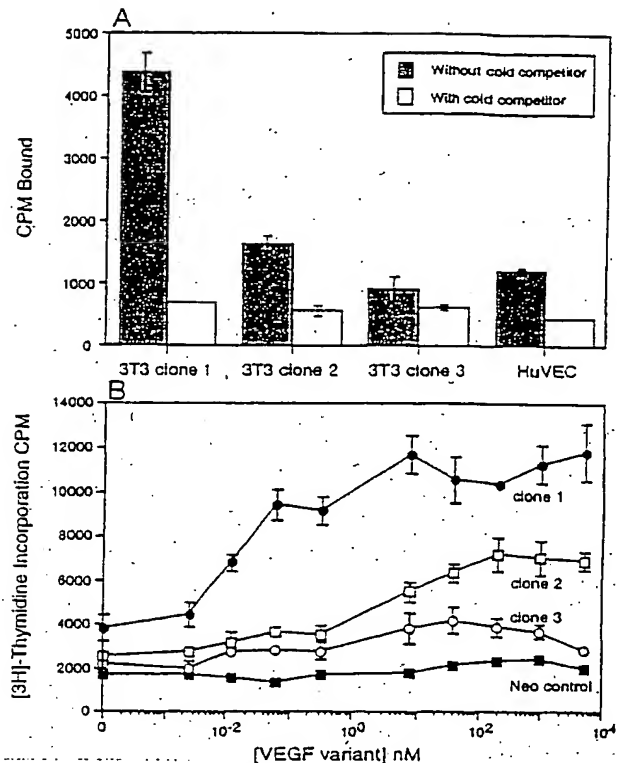


FIG. 6. 3T3 cells stably expressing varying amounts of KDR 1-7 (ECD)/Flt-4 (ICD) receptor respond to VEGF<sub>9-109</sub> with different values of EC<sub>50</sub> and maximal response. Panel A, three different 3T3 cell clones expressing varying amounts of the KDR 1-7 (ECD)/Flt-4 (ICD) were isolated and ranked according to the amount of functional binding sites for VEGF as determined by specific binding of [<sup>125</sup>I]-VEGF<sub>1-155</sub>. The same number of 3T3 cells from transfected clones 1, 2, and 3 or HuVEC were plated on the 24-well plate. The [<sup>125</sup>I]-VEGF<sub>1-155</sub> (0.1 nM) was added with (open bar) or without (filled bar) a 200-fold molar excess of cold VEGF<sub>1-155</sub> for 2 h and cells were washed and counted. Panel B, these same cell lines were treated with increasing concentrations of VEGF<sub>9-109</sub> and [<sup>3</sup>H]thymidine incorporation was measured. The three clones of 3T3 cells expressing KDR 1-7/Flt-4, NEO transfected control cells, and HuVEC were fasted and treated with serial dilutions of VEGF<sub>9-109</sub> for 18 h. Cells were pulsed with [<sup>3</sup>H]thymidine for 6 h before harvesting as described under "Experimental Procedures."

PDGF-α and -β receptors (25). Mutational analysis of nerve growth factor, another member of the cystine-knot family of dimeric hormones, shows a broad patch of residues involved in binding receptor that spans the interface between subunits (26).

**Requirements and Consequences for Receptor Dimerization**—Hormone-induced receptor dimerization is a general mechanism for activation of tyrosine kinase receptors (27). All receptors that bind cystine-knot hormone dimers are presumed to be activated by receptor dimerization (6). Here, gel filtration experiments provide *in vitro* evidence that VEGF binds two molecules of the extracellular domain of KDR. The dimerized complex appears to be very stable since excess VEGF is unable to dissociate the complex to 1:1 complex. This dimerization reaction is critical for signaling because the VEGF heterodimer, hV-1, with only one functional site is inactive in cell-based assays and antagonizes the action of wild-type VEGF. Receptor dimerization is also supported by the observation that cell-based assays show a bell-shaped dose-response curve with respect to VEGF. PDGF isoforms have been shown to induce

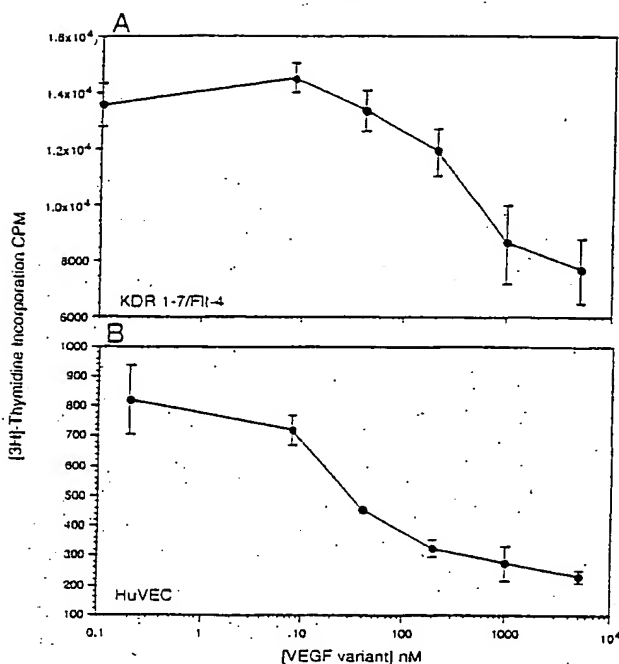


FIG. 7. The antagonistic effects of VEGF single site heterodimer (hV-1) on DNA synthesis of 3T3 cells stably expressing KDR 1-7 (ECD)/Flt-4 (ICD) (Panel A) or HuVEC (Panel B). Cells were incubated with either 0.1 nM VEGF<sub>1-109</sub> (for 3T3 cells) or 1 nM VEGF<sub>1-109</sub> (HuVEC) to induce 90% maximal incorporation of [<sup>3</sup>H]thymidine together with increasing concentrations of the hV-1.

dimerization of the extracellular domains of the PDGF- $\alpha$  and - $\beta$  receptors *in vitro* (28). Binding of the dimeric hormone, SCF, to the extracellular domain of the Kit receptor, a tyrosine kinase receptor of the IgG class, causes dimerization *in vitro* (29-31), and induces a bell-shaped dose-response curve *in vivo* (30).

Predimerized forms of KDR bind VEGF 100-fold more tightly than monomeric forms of KDR showing a strong avidity component in binding. Dimeric receptor fusion proteins, such as IgG fusions, or receptors bound to monoclonal antibodies are often used as convenient assay reagents for hormones and their variants. The data presented here show that there is a significant avidity component to binding in these fusions that affects the affinity constants. The avidity effect observed here is not the result of an alternate way that VEGF binds the dimeric KDR because alanine mutations in VEGF that are most disruptive to binding monomeric KDR are also the ones that most affect binding to dimeric KDR (Table III).

We observed that wild-type VEGF<sub>1-109</sub> binds about 100-fold more tightly than the single-site heterodimer to cells expressing KDR 1-7 (not shown). This suggests that receptors on cells may be loosely associated. Moreover, NIH 3T3 cells expressing larger numbers of VEGF receptors showed a higher basal level of [<sup>3</sup>H]thymidine incorporation in the absence of VEGF (Fig. 6B), suggesting that receptors on cells have an intrinsic ability to dimerize in the absence of ligand. Similar observations have been made for cells overexpressing various tyrosine kinase receptors, such as variants of the EGF receptor (27). Overexpression of the PDGF receptors can induce receptor autophosphorylation in the absence of ligand, and it is even possible to cross-link small amounts of the extracellular domains in the absence of PDGF (28). The fact we did not see evidence for dimerization by gel filtration of the ectodomains of KDR 1-7 or 1-3 in the absence of VEGF may only reflect the sensitivity of

the method and that the receptors have a much higher effective concentration on cells than in our solution experiments ( $\sim 1 \mu\text{M}$ ).

Deletion experiments showed that domains 2-3 of KDR are sufficient and necessary for high affinity binding of VEGF (Table I). Cells can signal when transfected with KDR domain 1-3 linked to Flt-4, even with low receptor expression, suggesting that domains 4-7 are not essential for signaling. We cannot rule out other roles for these domains; they may stabilize the signal transduction complex and/or provide for better display and expression of the receptor. Systematic deletion experiments have been conducted on at least four other tyrosine kinase receptors of the IgG class, and generally show that binding is dominated by IgG-like domains 2-3. Deletion experiments showed the first three of the five IgG-like domains in the Kit receptor are required for binding of SCF, but there is uncertainty regarding the role of domain 4 in signaling (30, 31). An antibody directed toward domain 4 blocked signaling in cells transfected with Kit, and deletion of domain 4 blocked signaling but not stem cell factor binding (30). In contrast, biophysical experiments (31) showed that Kit 1-3 can dimerize in solution with stem cell factor and both the enthalpy and free energy of binding were indistinguishable from Kit 1-5. In either case, both groups agree that the ligand-binding site for stem cell factor is contained in the first three IgG-like domains.

Deletion analysis of PDGF- $\alpha$  receptor, which contains five IgG-like domains, has shown that domains 2-3 are sufficient for binding PDGF isoforms although the presence of domain 1 has a small differential effect on binding PDGF-AA versus PDGF-BB (32). Deletion analysis of the fibroblast growth factor receptor, which contains three IgG-like domains, showed that domains 2-3 are sufficient for high affinity binding of fibroblast growth factor (33). Deletion experiments in Flt-1, which like KDR contains seven IgG-like domains, have shown that the VEGF-binding site is located among the first three IgG-like domains (34-36) and domain 2 of Flt-1 alone can bind VEGF tightly (23). Thus, domain 2 may play a dominant role in all five of these tyrosine kinase receptors that have IgG-like domains and may be general to the other members of this class.

**Mechanism-based Antagonists of VEGF**—Antagonists to VEGF may be very useful in preventing tumor angiogenesis and retinopathy diseases. Here, we have elucidated the functional requirements for receptor binding and activation and designed an antagonist, hV-1, for the proliferation of HuVEC cells based on this knowledge. The fact the IC<sub>50</sub> of hV-1 for inhibiting VEGF in HuVEC ( $\sim 20 \text{ nM}$ ) is  $\sim 100$ -fold higher than the EC<sub>50</sub> of VEGF stimulating growth ( $\sim 0.2 \text{ nM}$ ) likely reflects the avidity effect described above. We believe the hV-1 antagonize VEGF stimulation of HuVEC by blocking the dimerization of KDR since KDR is more important for signaling mitogenesis. However, the hV-1 does bind Flt-1 with near wild-type affinity and we are currently looking at its ability to activate Flt-1. Alanine scanning of both receptor-binding sites on VEGF suggests that the binding sites for KDR and Flt-1 overlap and are not identical (9, 23, 37). Based on these results it should also be possible to design receptor specific antagonists and further elucidate the functions of the two receptors. Overall, these studies provide a basis from which we can produce new analogs of VEGF to both probe its biology and generate new and potent therapeutics.

**Acknowledgments**—We thank Toni Klassen and Jin Kim for mAbs to KDR, Hans Christinger for providing purified VEGF, Napoleone Ferrara for providing the plasmid pHEBO23-KDR-IgG and pHEBO23-Flt-1-IgG, Jennifer Singh, Richard DeMarco, Michael Clasen for technical support, the DNA Synthesis Group at Genentech for oligonucleotides, and David Wood for the graphics.

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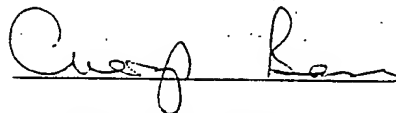
IN THE MATTER OF Australian Patent  
Application Serial No. 696764 by Human  
Genome Sciences, Inc.

-and-

IN THE MATTER OF Opposition thereto by  
Ludwig Institute for Cancer Research

THIS IS Exhibit PAWR-9  
referred to in the Statutory Declaration  
of Peter Adrian Walton Rogers  
made before me

DATED this 12<sup>th</sup> Day of November, 2001



(Signature of Witness)

Medical Practitioner

## EXEMPLARY PORTIONS OF HGS EVIDENCE DEVOTED TO EXPERIMENTATION TO TRY TO COMPLETE THE VEGF2 INVENTION

Further experimentation suggested by Dr. Mattick in order to understand the VEGF2 invention or how to make it work:

AJM1 3.15	Computer analysis of sequence for signal peptide.
AJM1 3.16-19	Expression of protein from DNA
AJM1 3.18	Overcoming obstacle of realizing that protein doesn't express because signal sequence is missing or faulty.
AJM1 3.33	Experimentation to express a biologically active VEGF2
AJM1 3.34	Experimentation to obtain VEGF2 polynucleotide sequences.
AJM1 4.3-4.13	Experimentation to express VEGF2 notwithstanding "the fact that the signal sequence information was incomplete" in the opposed application.
AJM1 4.15-4.16	Confirming biological activity, suggestion of collaboration
AJM1 4.64-4.68	Experimentation to produce fragments analogues and derivatives, and identification of such molecules
AJM1 4.77	Expression of incomplete sequence
AJM1 4.82-4.83	Production of antibodies and determination of VEGF2 activity

Further experimentation suggested by Dr. Gamble in order to understand the VEGF2 invention or how to make it work:

AJG1 5.14-5.19	Assaying for angiogenic properties
AJG1 7.9-7.10	Testing for <i>in vivo</i> or <i>in vitro</i> activity
AJG1 7.11	Distinguishing between growth factors
AJG1 7.20	Antibody production

AJG1 7.35

Experimentation to produce fragments  
analogues and derivatives

Further experimentation suggested by Dr. Hayward in order to understand the VEGF2 invention or how to make it work:

ANH1 3.11-3.12	Designing suitable hybridization conditions
ANH1 3.19-3.21, 4.20	Use of heterologous signal sequence
ANH1 3.25	5' end cloning
ANH1 3.27	Testing for a biological activity
ANH1 3.29	Testing VEGF2 in proliferation, angiogenesis and wound healing assays
ANH1 3.34	Testing of receptor binding
ANH1 4.4	Activity assays
ANH1 4.26-4.27	Redesigning Example 2 in the application in order to attempt to achieve the results that were reported

Further experimentation suggested by Dr. Rapoport in order to understand the VEGF2 invention or how to make it work: (essentially entire declaration, devoted to identifying signal peptide defect in patent application, and then trying to overcome it.)

Further experimentation suggested by Dr. Aaronson in order to understand the VEGF2 invention or how to make it work:

ASA1 at 16	Engineering a heterologous signal sequence
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Further experimentation suggested by Dr. Power in order to understand the VEGF2 invention or how to make it work: (Entire Declaration devoted to signal peptide experiments which are not based on the application's teachings.)

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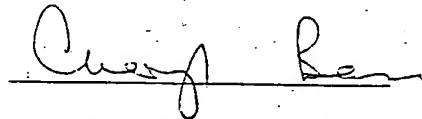
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-and-

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THIS IS Exhibit PAWR-10  
referred to in the Statutory Declaration  
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made before me

DATED this 12<sup>th</sup> Day of November, 2001



(Signature of Witness)

Medical Practitioner

## EXEMPLARY PORTIONS OF HGS EVIDENCE DEVOTED TO CATALOGUING PREDICTIONS IN THE PATENT APPLICATION

Cataloging by Dr. Mattick of statements, unsupported predictions, and other unsupported excerpts of the HGS application:

- AJM1 3.25, 3.30-3.31 (Alleged uses of VEGF2 - no supporting data)
- AJM1 3.32 and JSM-4 Table 1 (essentially a table of contents for application.)
- AJM1 4.3 and 4.5-4.11 (overcoming signal sequence issue)
- AJM1 4.18-4.24 (biological properties of VEGF2 and theoretical uses)
- AJM1 4.30 and 4.39 (hybridization conditions and control thereof)
- AJM1 4.67 (testing for activity)
- AJM1 4.70 and 4.73 (gene therapy and treatments)
- AJM1 4.77 (heterologous signal sequence usage)
- AJM1 4.82-4.83 (antibody production)
- AJM1 4.108 (antagonists)

Cataloging by Dr. Gamble of predictions and other unsupported excerpts of the HGS application:

- AJG1 6.5 (prediction of processing)
- AJG1 6.6 (expression systems)
- AJG1 6.7 (activity assays)
- AJG1 6.8 - 6.8.11 (list like Mattick)
- AJG1 7.11 (assays for fragments, etc)
- AJG1 7.15-7.18 (angiogenic assays)
- AJG1 7.20 (generation of antibodies)
- AJG1 7.24 (prediction of processing)
- AJG1 7.35 (generation of fragments)

Cataloging by Dr. Hayward of predictions and other unsupported excerpts of the HGS application:

ANH1 3.6 (activities fo VEGF2 and/or fragments, etc.)  
ANH1 3.11 (hybridization conditions)  
ANH1 3.15 (expectation of secretion)  
ANH1 3.19-3.21 (use of heterologous signal sequence)  
ANH1 3.22 (processing)  
ANH1 3.27-3.30 (activities and uses of VEGF2)  
ANH1 3.34 & 3.35 (binding of receptors)  
ANH1 3.37 (uses of fragments and antibodies)  
ANH1 4.4 (assays and activities)

Cataloging by Dr. Rapoport of predictions and other unsupported excerpts of the HGS application:

ATR1 at 11 (prediction of processing)  
ATR1 at 12 (heterologous signal sequence)  
ATR1 at 18 (prediction of a secreted growth factor)

Cataloging by Dr. Aaronson of predictions and other unsupported excerpts of the HGS application:

ASA1 at 5 (prediction of secretion)  
ASA1 at 6 (proteolytic processing)  
ASA1 at 16 (heterologous signal sequence)

AUSTRALIA

*Patents Act 1990*

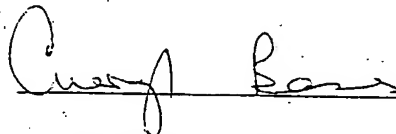
IN THE MATTER OF Australian Patent  
Application Serial No. 696764 by Human  
Genome Sciences, Inc.

-and-

IN THE MATTER OF Opposition thereto by  
Ludwig Institute for Cancer Research

THIS IS Exhibit PAWR-11  
referred to in the Statutory Declaration  
of Peter Adrian Walton Rogers  
made before me

DATED this 12<sup>th</sup> Day of November, 2001



(Signature of Witness)

Medical Practitioner



US006221839B1

(12) **United States Patent**  
Alitalo et al.

(10) Patent No.: **US 6,221,839 B1**  
(45) Date of Patent: **Apr. 24, 2001**

(54) **FLT4 LIGAND AND METHODS OF USE**

(75) Inventors: Kari Alitalo, Espoo; Vladimir Joukov, Helsinki, both of (FI)

(73) Assignees: Helsinki University Licensing Ltd. Oy, Helsinki (FI); Ludwig Institute for Cancer Research, New York

(\*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: 08/510,133

(22) Filed: Aug. 1, 1995

(51) Int. Cl.<sup>7</sup> ..... A61K 38/18; C07K 14/475

(52) U.S. Cl. .... 514/12; 514/2; 530/399

(58) Field of Search ..... 530/399; 514/2, 514/21

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(57) **ABSTRACT**

Provided are ligands for the receptor tyrosine kinase, Flt4. Also provided are cDNAs and vectors encoding the ligand, pharmaceutical compositions and diagnostic reagents.



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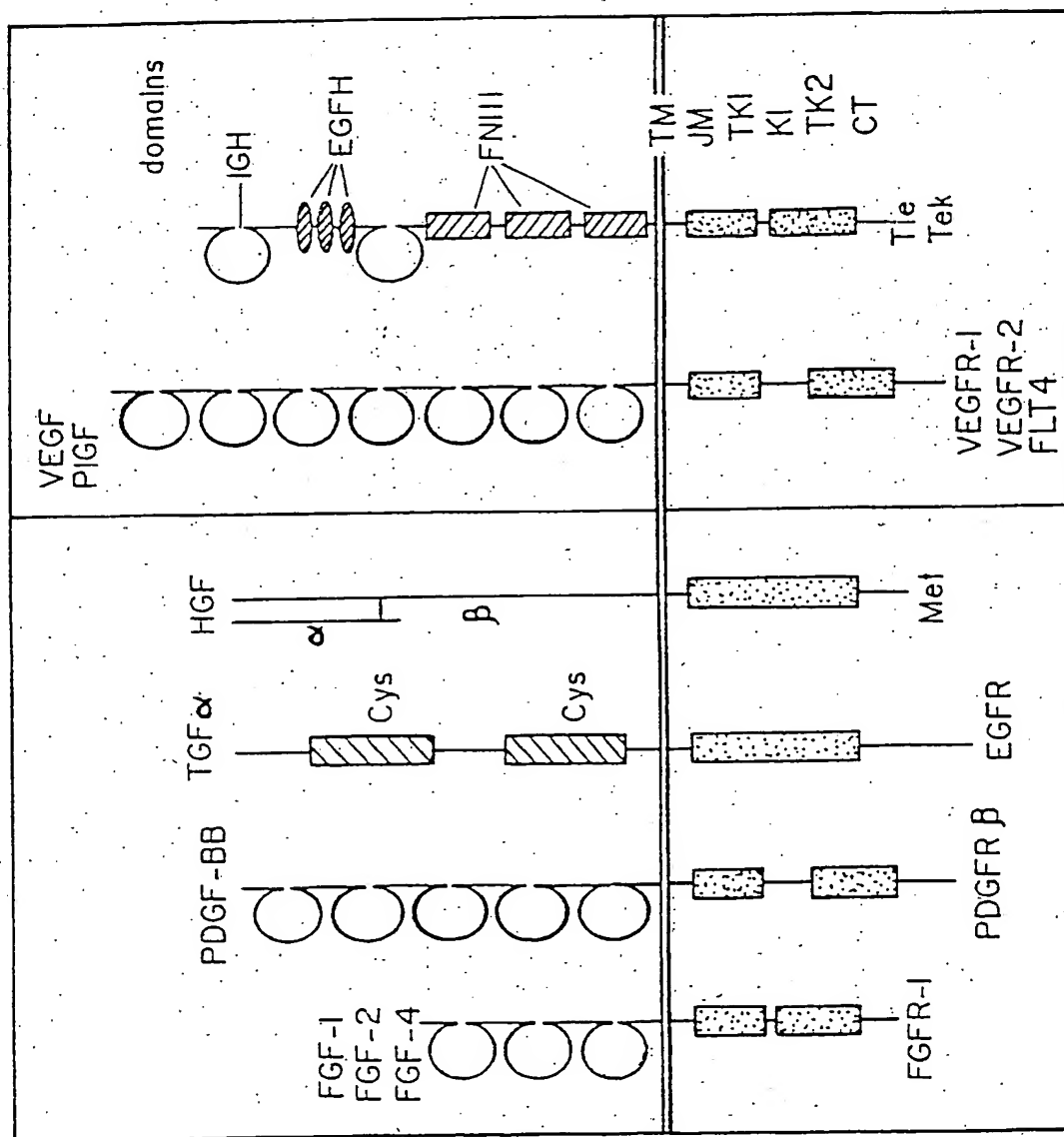


FIGURE 1

FIGURE 2A

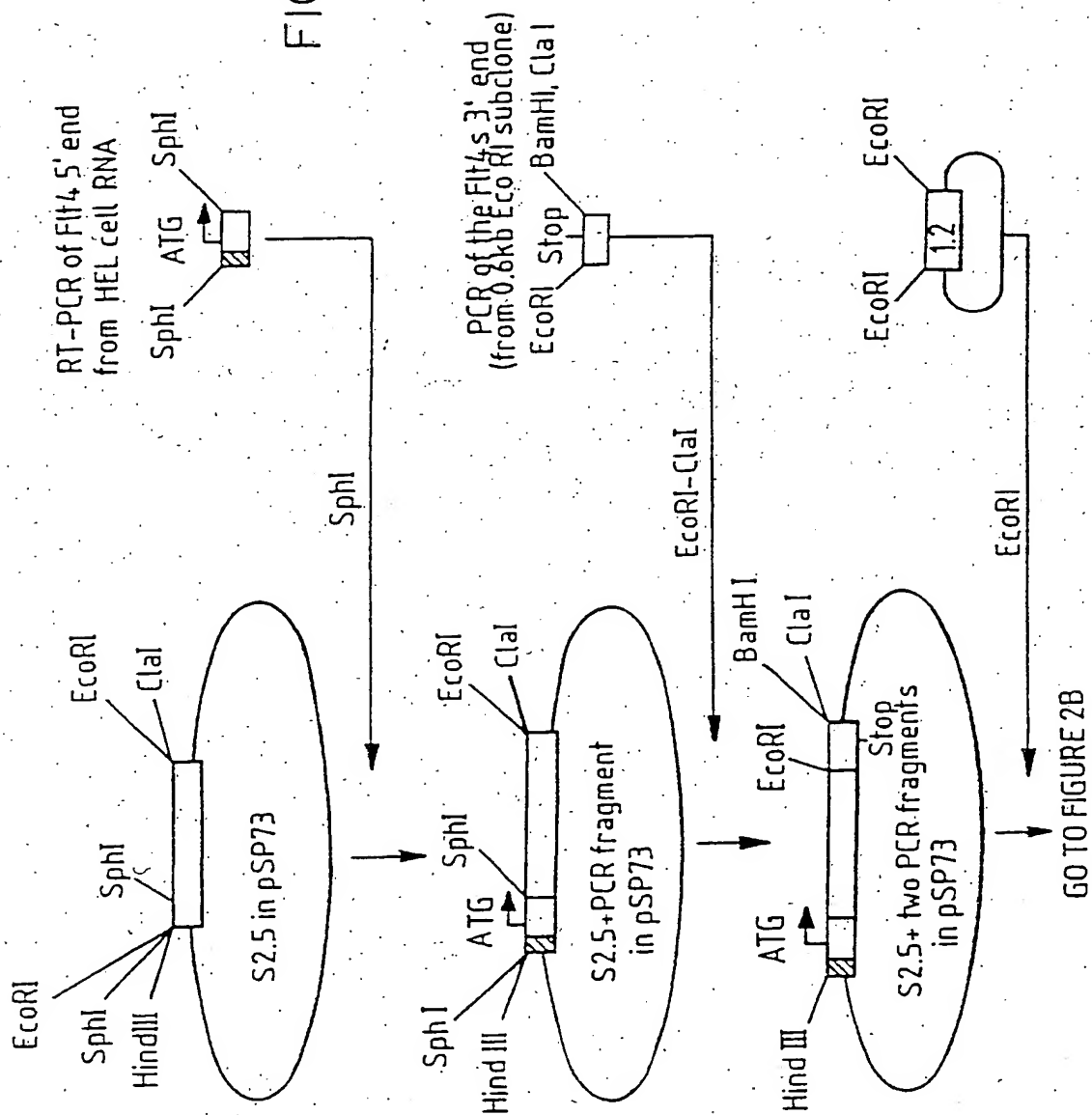
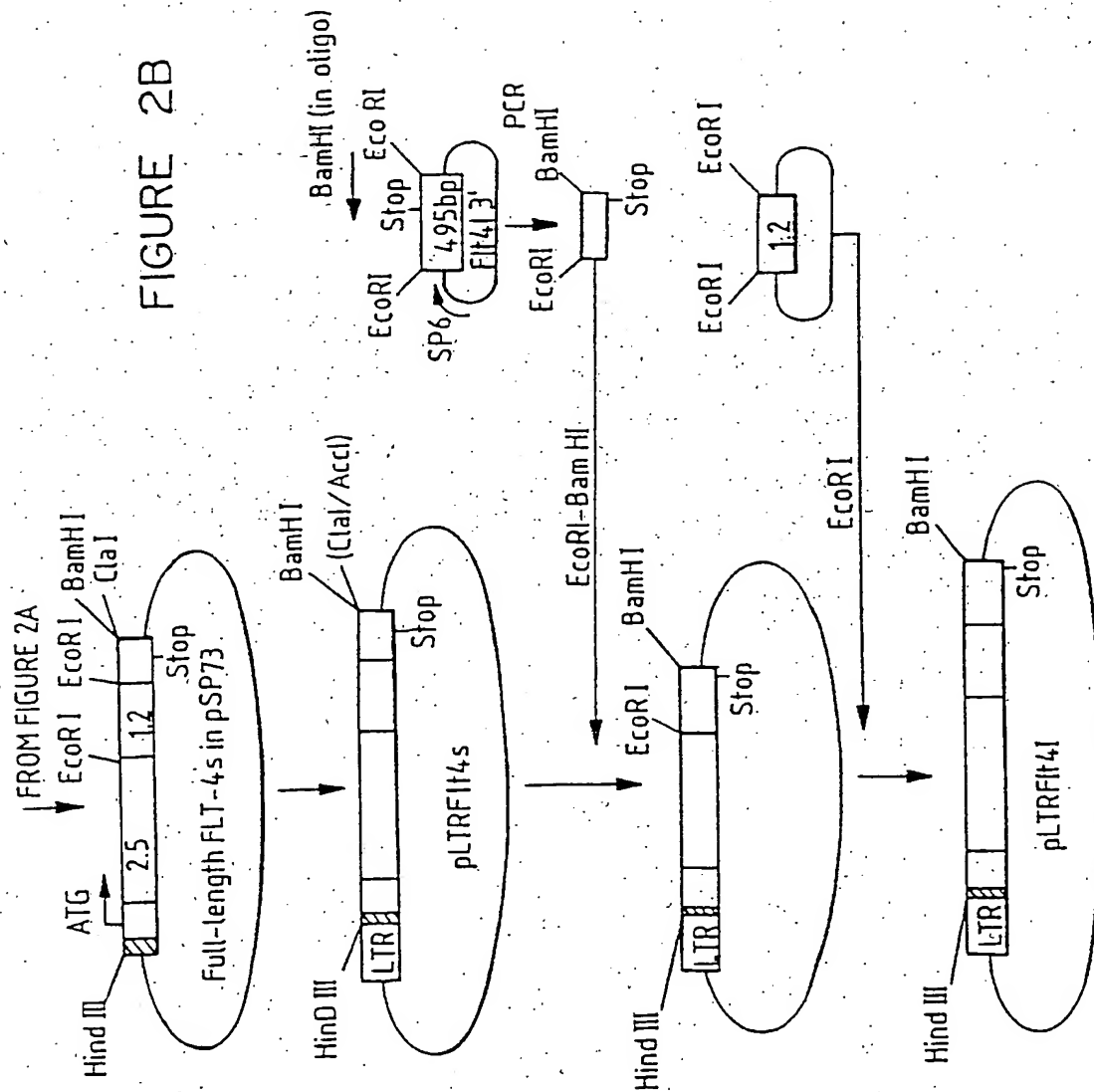


FIGURE 2B



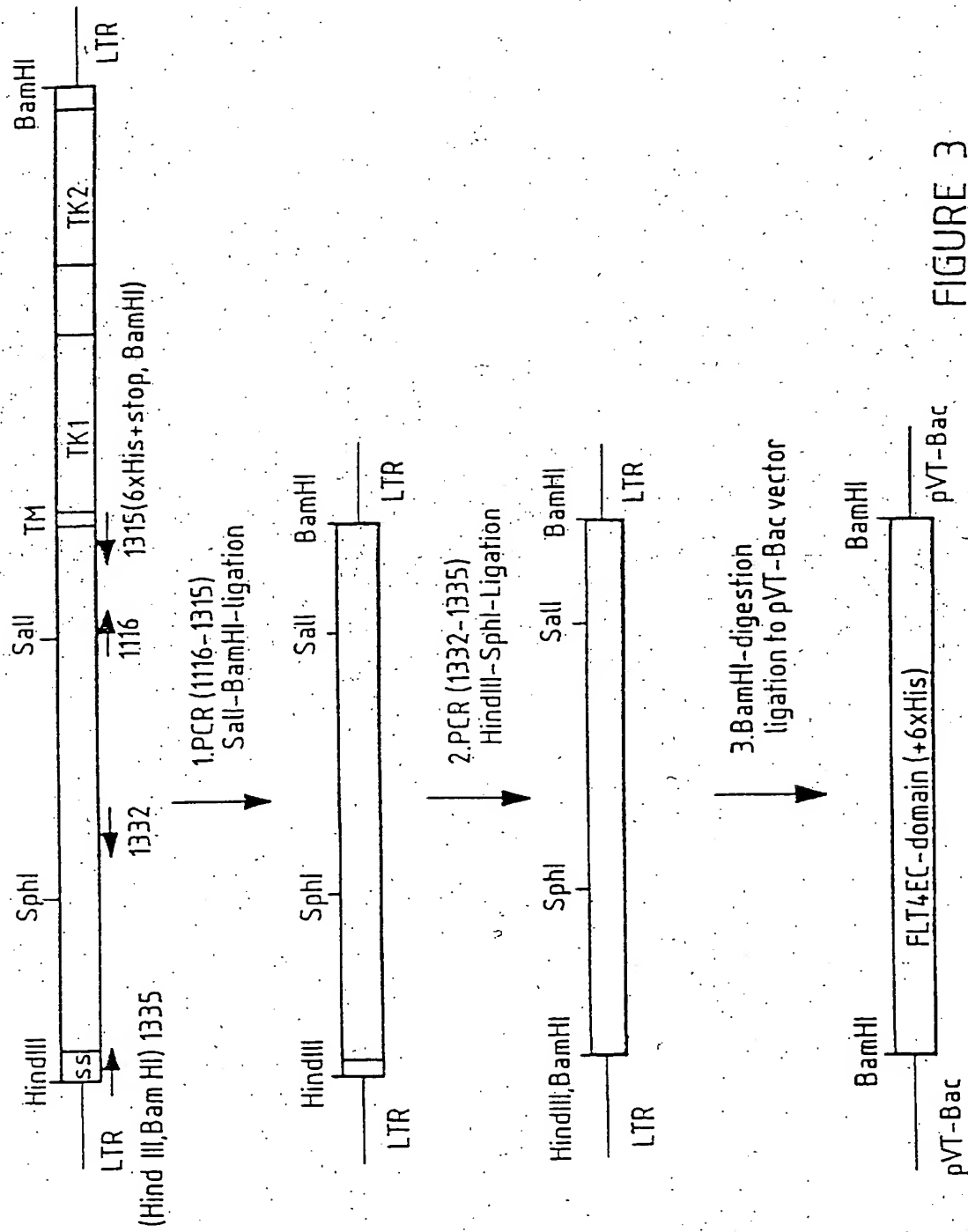


FIGURE 3



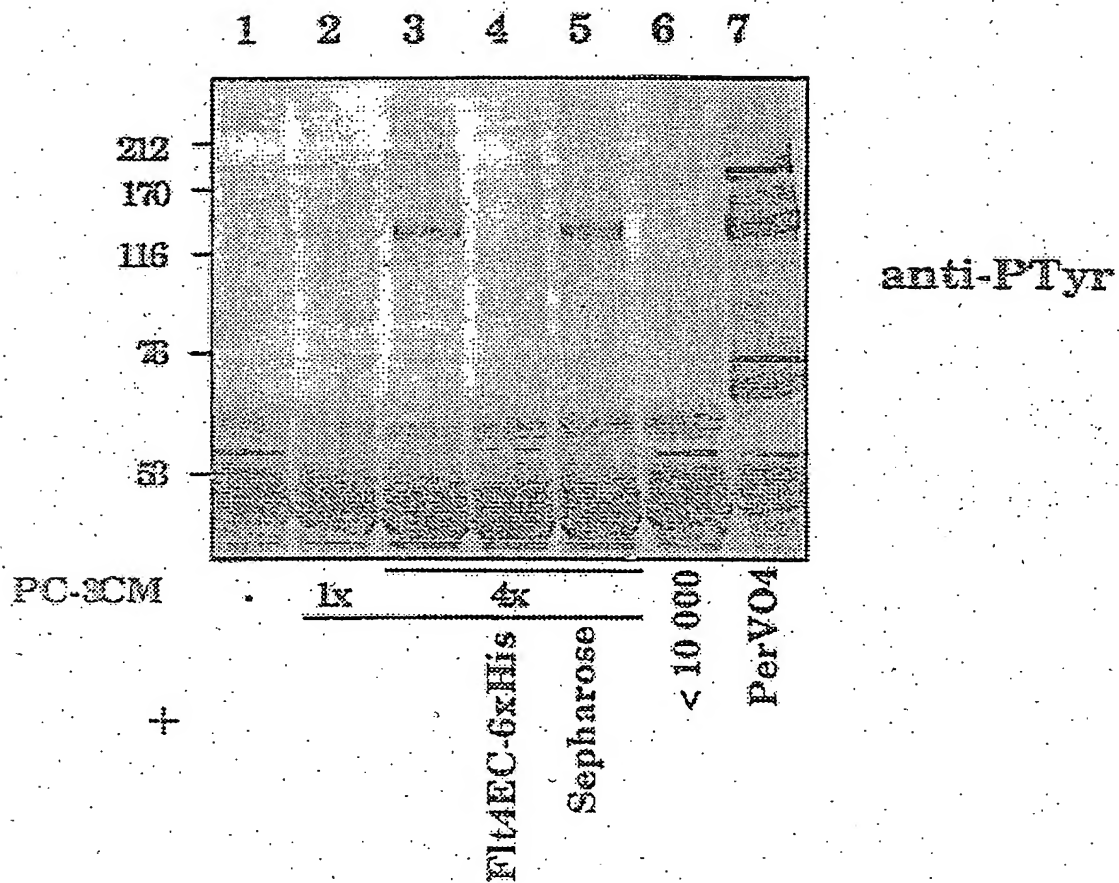


FIGURE 4

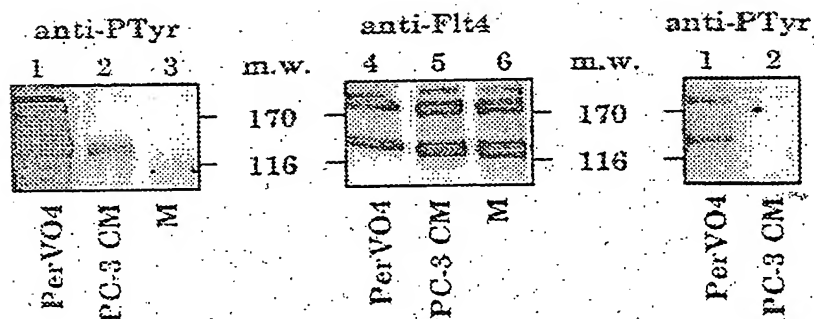


FIGURE 5A

FIGURE 5B

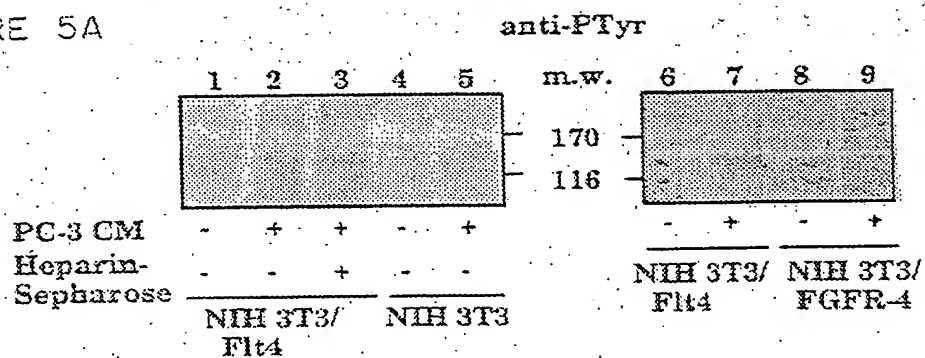


FIGURE 5C

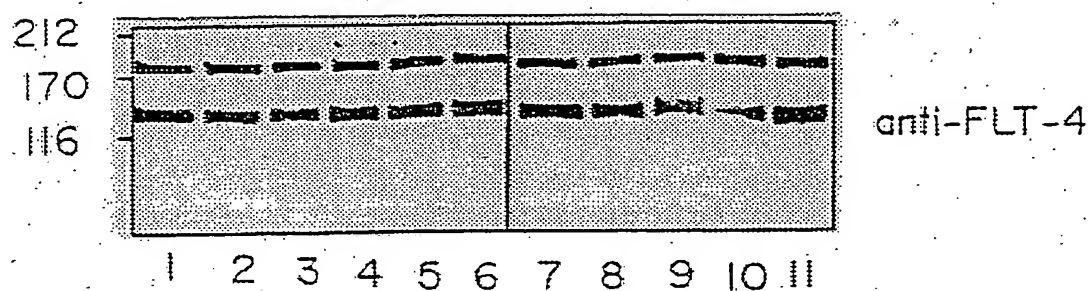


FIGURE 6A

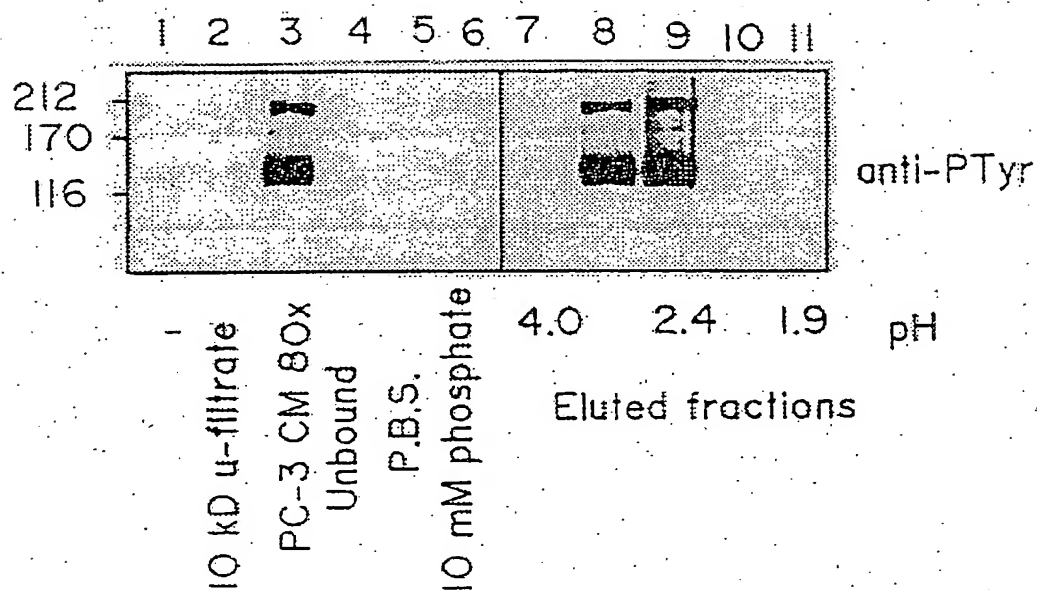


FIGURE 6B

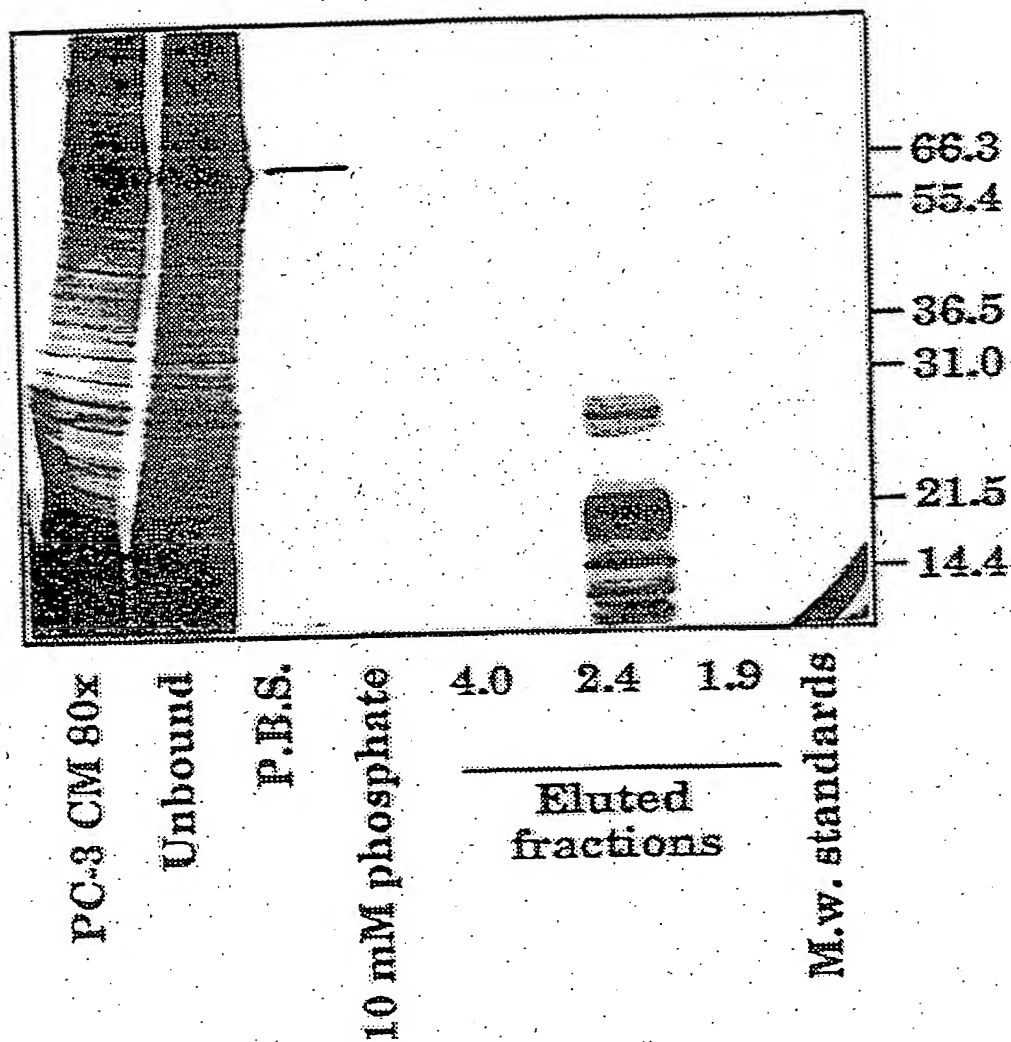


FIGURE 7

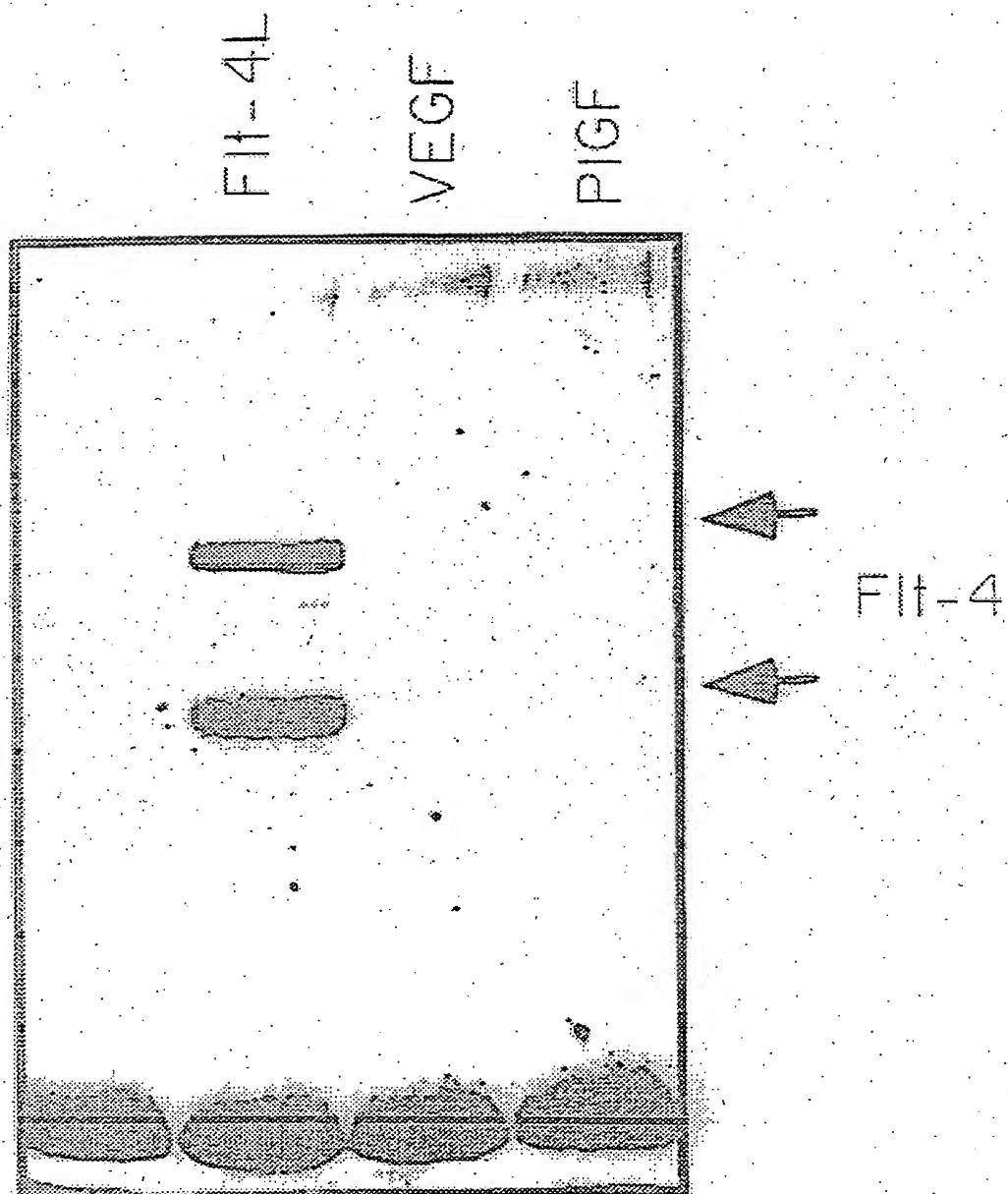


FIGURE 8

MetThrValLeuTyrProGluTyr  
GAGCAGTTACGGTCTGTGTCCAGTGTAGATGAACATCATGACTGTACTCTACCCAGAAATAT  
10 30 50

TrpLysMetTyrLysCysGlnLeuArgLysGlyGlyTyrGlnHisAsnArgGluGlnAla  
TGGAAAATGTACAAAGTGTACAGCTAAGGAAAGGAGGCTGGCAACATAACAGAGAACAGGCC  
70 90 110

AsnLeuAsnSerArgThrGluGluThrIleLysPheAlaAlaAlaHisTyrAsnThrGlu  
AACCTCAACTCAAGCACAGACAGAGACTATAAAATTTGCTGCAGCACATTATAATACAGAG  
130 150 170

IleLeuLysSerIleAspAsnGluTrpArgLysThrGlnCysMetProArgGluValCys  
ATCTTGAAAAGTATTGATAATGAGTGGAGAAAGACTCAATGCATGCCACGGGAGGTGTGT  
190 210 230

IleAspValGlyLysGluPheGlyValAlaThrAsnThrPhePheLysProProCysVal  
ATAGATGTGGGAAGGAGTTTGGAGTCGCGACAAACACCTTCTTTAAACCTCCATGTGTG  
250 270 290

SerValTyrArgCysGlyGlyCysCysAsnSerGluGlyLeuGlnCysMetAsnThrSer  
TCCGTCTACAGATGTGGGGGTTGCTGCAATAGTGAGGGGCTGCAGTGCATGAACACCAGC  
310 330 350

FIGURE 9A

ThrSerTyrLeuSerLysThrLeuPheGluIleThrValProLeuSerGlnGlyProLys  
ACGAGCTACCTCAGCAAGACGTTATTGTGAATTAACAGTGCCTCTCTCAAGGCCCAAA  
370 390 410

ProValThrIleSerPheAlaAsnHisThrSerCysArgCysMetSerLysLeuAspVal  
CCAGTAACAATCAGTTTGTGCAATCACACTTCCTGCCGATGCATGTCTAAACTGGATGTT  
430 450 470

TyrArgGlnValHisSerIleIleArgArgSerLeuProAlaThrLeuProGlnCysGln  
TACAGACAAAGTTCATTCCATTATTAGACGTTCCCTGCCAGCAACACTACCACAGTGTCTAG  
490 510 530

AlaAlaAsnLysThrCysProThrAsnTyrMetTrpAsnAsnHisIleCysArgCysLeu  
GCAGCGAACAAGACCTGCCCCCACCATTACATGTGGAATAATCACATCTGCAGATGCCCTG  
550 570 590

AlaGlnGluAspPheMetPheSerSerAspAlaGlyAspAspSerThrAspGlyPheHis  
GCTCAGGAAGATTTTATGTTTTCCTCGGATGCTGGAGATGACTCAACAGATGGATTCCAT  
610 630 650

AspIleCysGlyProAsnLysGluLeuAspGluGluThrCysGlnCysValCysArgAla  
GACATCTGTGGACCAACAAGAGAGCTGGATGAAGAGACCCTGTCTAGTGTCTGCAGAGCG  
670 690 710

FIGURE 9B

GlyLeuArgProAlaSerCysGlyProHisLysGluLeuAspArgAsnSerCysGlnCys  
GGGCTTCGGCCTGCCAGCTGTGGACCCCAAGAACTAGACAGAAACTCATGCCAGTGT  
730 750 770

ValCysLysAsnLysLeuPheProSerGlnCysGlyAlaAsnArgGluPheAspGluAsn  
GTCTGTAAAAACAACACTCTTCCCCAGCCCAATGTGGGGCCAAACCGAGAATTGTATGAAAAAC  
790 810 830

ThrCysGlnCysValCysLysArgThrCysProArgAsnGlnProLeuAsnProGlyLys  
ACATGCCAGTGTGTATGTAAAGAACCTGCCCCCAGAAATCAACCCCTAAATCCTGGAAAAA  
850 870 890

CysAlaCysGluCysThrGluSerProGlnLysCysLeuLeuLysGlyLysLysPheHis  
TGTGCCTGTGAATGTACAGAAAGTCCACAGAAATGCTTGTTAAAGGAAAGAACTTCCAC  
910 930 950

HisGlnThrCysSerCysTyrArgArgProCysThrAsnArgGlnLysAlaCysGluPro  
CACCAAAACATGCAGCTGTTACAGACGGCCATGTACGAACCCGCCAGAGGCTTGTGAGCCA  
970 990 1010

GlyPheSerTyrSerGluGluValCysArgCysValProSerTyrTrpLysArgProGln  
GGATTTTCATATAGTGAAGAAGTGTGTCGTTGTGTCCCTTCATATATTGGAAGAACCCACAA  
1030 1050 1070

MetSerEnd

ATGAGCTAAGATTGTACTGTTTCCAGTTCATCGATTTTCTATTATGGAAGAACTGTGTG  
1090 1110 1130

FIGURE 9C

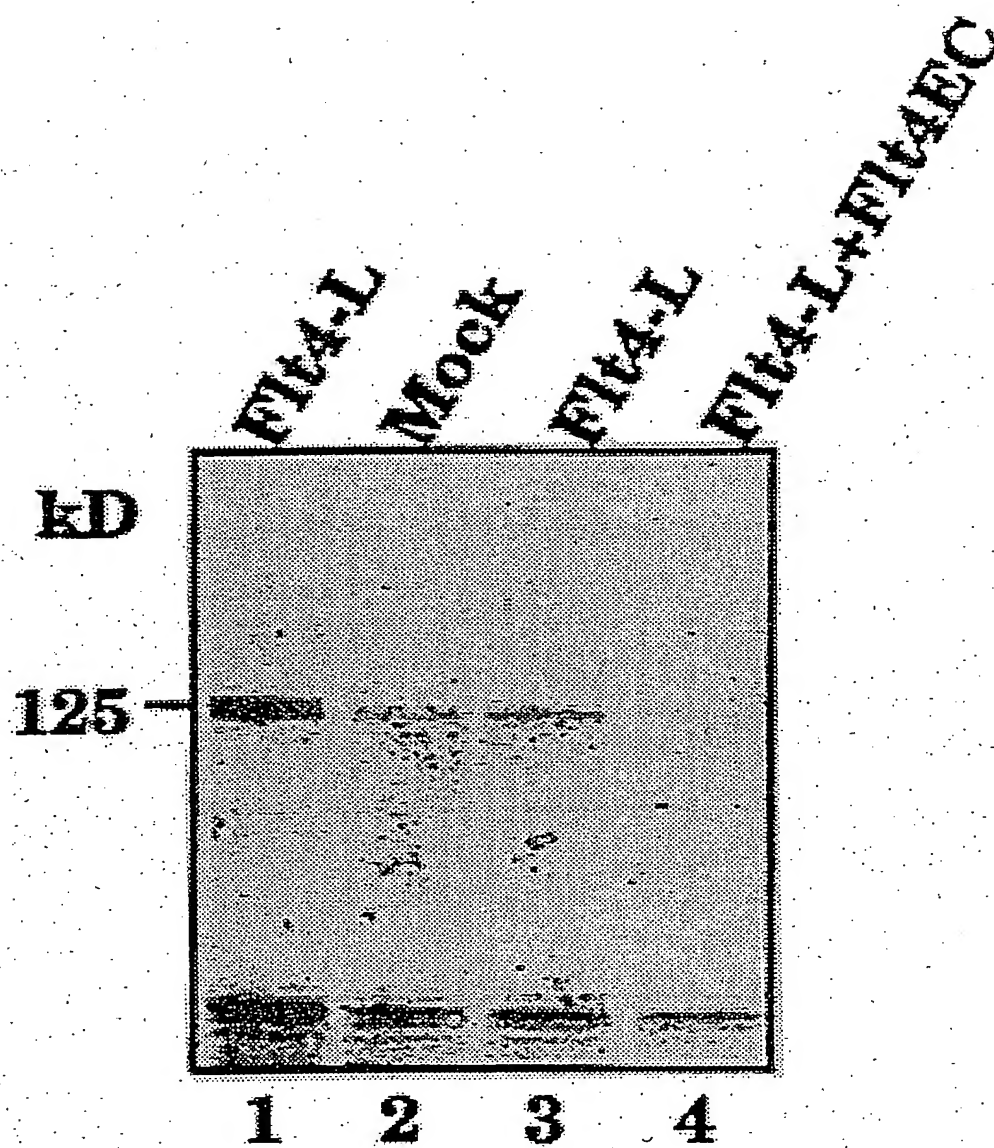


1 50  
PDGF-A MRTWACLLL LGCGLAHL AEEAIPREL IERLARSQIH SIRDQLRLLE  
PDGF-B MNRCAW LFL SLCCYLRLVS AEGDPIPEEL YEMLSHDSIR SFDDLQRLIH  
PLGF ..... MP VMRLFPCFLQ LLAGLAL  
VEGF ..... MNFLLSWVH WSLALLLYLH  
FLT4-L ..... MTVLYPEYWK MYKQQLRKGG 100  
51  
PDGF-A IDSVGAEDAL ETSRAHGS AINHVEKRP VPIRRKRSI .....EEAIP  
PDGF-B GDP.GEEDGA ELDLNMTRSH SGGELES ..... LARGRRSLG SLTIAEPAMI  
PLGF PAVPPQQWAL SA ..... GNGSSEVEV P.FQEVWG .....R  
VEGF HAKWSQAAPM AE ..... GGGQNHHEV K.FMDVYQ .....R  
FLT4-L WQHNREQANL NSRTEETIKF AAHYNTEIL KSIDNEWK .....K 150  
101  
PDGF-A AVCKTRTVIY EIPRSQVDPT SANFLIWPCC VEVKRCIGCC NTSSVKCQPS  
PDGF-B AECKTRTEVF EISRRIDRT NANFLVWPPC VEVQRCSGCC NNRNVQCRPT  
PLGF SYCRALERLV DVVSEY..PS EVEHMFSPSC VSLIRCIIGCC GDENLHCVPV  
VEGF SYCHPIETLV DIFQEY..PD EIEYIFKPS VPLMRCGGCC NDEGLECVPT  
FLT4-L TCCMPREVC I DVGKEF..GV ATNTFFKPPC VSVYRCGGCC NSEGLQCMNT 200  
151  
PDGF-A RVHRSVKVA KVEYVRKKPK LKEVQVRLEE FILECAC .....AT  
PDGF-B QVQLRPVQVR KIEIVRKKPI FKKATVTLED HLAACK .....ETVAAARPVT  
PLGF ETANVTMQLL KIRSG..DRP..SYVELTFSQ HVRCECRPLR EKMKPERC..  
VEGF EESNITMQIM RIKPH..QGQ..HIGEMSFLQ HNKCECRPKK DRARQENP..  
FLT4-L STSYLSKTLF EITVPLSQGP..KPVITISFAN HTSCPCMSKL DVYRQVHSII

FIGURE 10A

201 250  
PDGF-A : SNLNPDHR EEETDVR .....  
PDGF-B RSPGGSQEQR AKTPQTRVTI RTVRVRRPPK GKHRKFKHTH DKTALKETLG  
PlGF ..... GDAVPRR .....  
VEGF ..... CGPCSERRKH LFVQDPQTCK CCKNTDSRC KARQLELNER  
FLT4-L RRSLPATLPQ CQAANKTCPT NYMWNHICR CLAQEDFMFS SDAGDDSTDG 300  
251  
PDGF-A .....  
PDGF-B A .....  
PlGF .....  
VEGF ..... TCRCDKPRR .....  
FLT4-L FHDICGPNKE LDEETCQCVC RAGLRPASC GPHKELDRNSC QCVCCKNKLFP 350  
301  
PDGF-A .....  
PDGF-B .....  
PlGF .....  
VEGF .....  
FLT4-L SQCGANREFD ENTQCQCCKR T CPRNQPLNP GKACECTES PQKCLLKGGK 395  
351  
PDGF-A .....  
PDGF-B .....  
PlGF .....  
VEGF .....  
FLT4-L FHHQTCSCYR RPCTNRQKAC EPGFSYSEEV CRCVPSYWKR PQMS

FIGURE 10B

**FIGURE II**

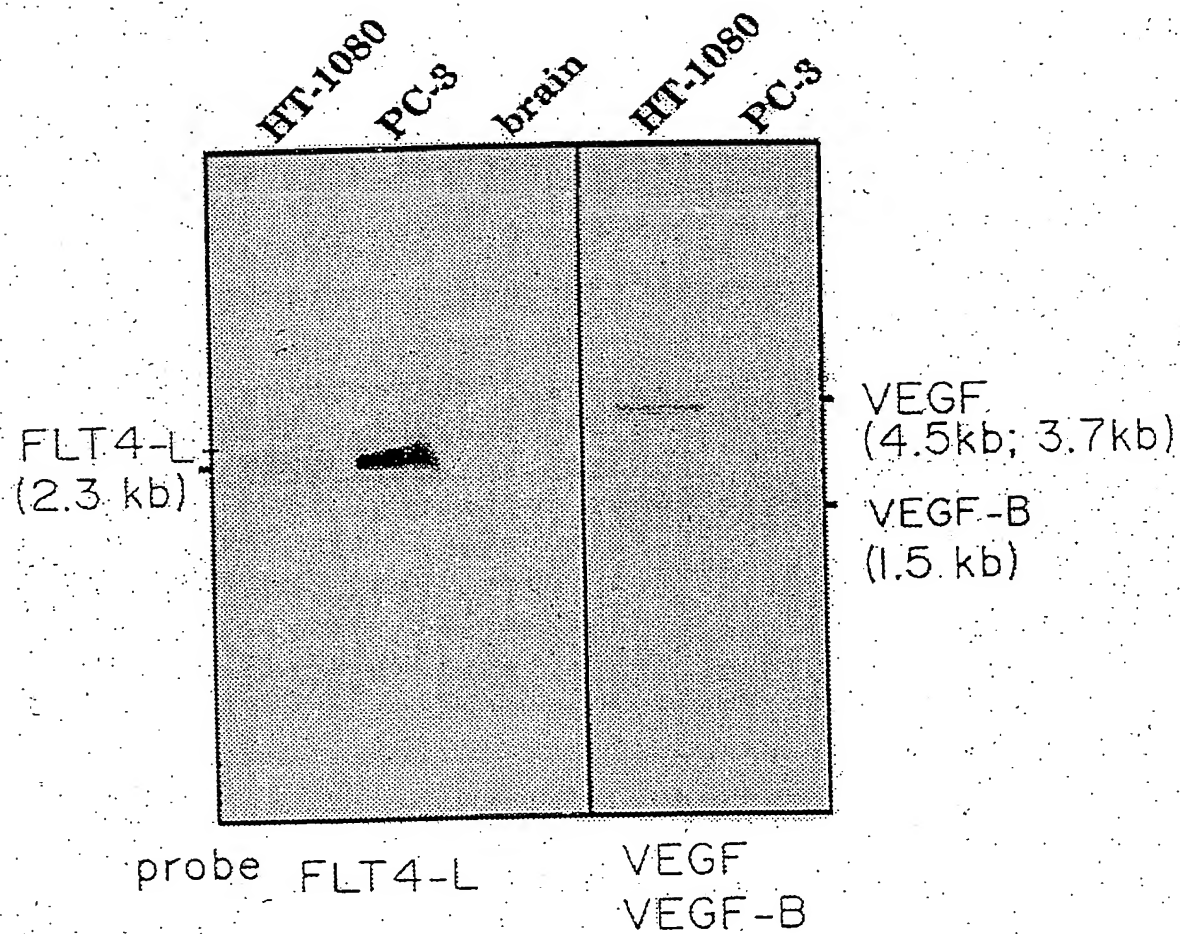


FIGURE 12

## FLT4 LIGAND AND METHODS OF USE

## FIELD OF THE INVENTION

The present invention generally relates to the field of genetic engineering and more particularly to growth factors for endothelial cells and growth factor genes.

## BACKGROUND OF THE INVENTION

Developmental growth, the remodelling and regeneration of adult tissues as well as solid tumor growth, can only occur when accompanied by blood vessel formation. Angioblasts and hematopoietic precursor cells differentiate from the mesoderm and form the blood islands of the yolk sac and the primary vascular system of the embryo. The development of blood vessels from these early (in situ) differentiating endothelial cells is termed vasculogenesis. Major embryonic blood vessels are believed to arise via vasculogenesis, whereas the formation of the rest of the vascular tree is thought to occur as a result of vascular sprouting from pre-existing vessels, a process called angiogenesis, Risau, et al., *Devel. Biol.*, 125: 441-450 (1988).

Endothelial cells give rise to several types of functionally and morphologically distinct vessels. When organs differentiate and begin to perform their specific functions, the phenotypic heterogeneity of endothelial cells increases. Upon angiogenic stimulation, endothelial cells may re-enter the cell cycle, migrate, withdraw from the cell cycle and subsequently differentiate again to form new vessels that are functionally adapted to their tissue environment. Endothelial cells undergoing angiogenesis degrade the underlying basement membrane and migrate, forming capillary sprouts that project into the perivascular stroma. Ausprunk, et al., *Microvasc. Rev.*, 14: 51-65 (1977). Angiogenesis during tissue development and regeneration depends on the tightly controlled processes of endothelial cell proliferation, migration, differentiation and survival. Dysfunction of the endothelial cell regulatory system is a key feature of many diseases. Most importantly, tumor growth and metastasis have been shown to be angiogenesis dependent. Folkman, et al., *J. Biol. Chem.*, 267:10931-10934 (1992).

Key signals regulating cell growth and differentiation are mediated by polypeptide growth factors and their transmembrane receptors, many of which are tyrosine kinases. Autophosphorylated peptides within the tyrosine kinase insert and carboxyl-terminal sequences of activated receptors are commonly recognized by kinase substrates involved in signal transduction for the readjustment of gene expression in responding cells. Several families of receptor tyrosine kinases have been characterized. Van der Geer, et al., *Ann. Rev. Cell Biol.*, 10:251-337 (1994). The major growth factors and receptors transducing angiogenic stimuli are schematically shown in FIG. 1.

Fibroblast growth factors are also known to be involved in the regulation of angiogenesis. They have been shown to be mitogenic and chemotactic for cultured endothelial cells. Fibroblast growth factors also stimulate the production of proteases, such as collagenases and plasminogen activators, and induce tube formation by endothelial cells. Saksela, et al., *Ann. Rev. Cell Biol.*, 4:93-126 (1988). There are two general classes of fibroblast growth factors, FGF-1 and FGF-2, both of which lack conventional signal peptides. Both types have an affinity for heparin and FGF-2 is bound to heparin sulfate proteoglycans in the subendothelial extracellular matrix from which it may be released after injury. Heparin potentiates the stimulation of endothelial cell proliferation by angiogenic FGFs, both by protecting against

denaturation and degradation and dispersing the FGFs. Cultured endothelial cells express the FGF-1 receptor but no significant levels of other high-affinity fibroblast growth factor receptors.

Among other ligands for receptor tyrosine kinases, the platelet derived growth factor, PDGF-BB, has been shown to be weakly angiogenic in the chick chorioallantoic membrane. Risau, et al., *Growth Factors*, 7:261-266 (1992). Transforming growth factor  $\alpha$  (TGF $\alpha$ ) is an angiogenic factor secreted by several tumor cell types and by macrophages. Hepatocyte growth factor (HGF), the ligand of the c-met proto-oncogene-encoded receptor, is also strongly angiogenic.

Recent evidence shows that there are endothelial cell specific growth factors and receptors that may be primarily responsible for the stimulation of endothelial cell growth, differentiation and certain differentiated functions. The best studied of these is vascular endothelial growth factor (VEGF), a member of the PDGF family. Vascular endothelial growth factor is a dimeric glycoprotein of disulfide-linked 23 kDa subunits, discovered because of its mitogenic activity toward endothelial cells and its ability to induce vessel permeability (hence its alternative name vascular permeability factor). Other reported effects of VEGF include the mobilization of intracellular calcium, the induction of plasminogen activator and plasminogen activator inhibitor-1 synthesis, stimulation of hexose transport in endothelial cells, and promotion of monocyte migration in vitro. Four VEGF isoforms encoded by distinct mRNA splice variants appear to be equally capable of stimulating mitogenesis in endothelial cells. However, each has a different affinity for cell surface proteoglycans, which behave as low affinity receptors for VEGF. The 121 and 165 amino acid isoforms of VEGF are secreted in a soluble form, whereas the isoforms of 189 and 206 amino acid residues remain cell surface associated and have a strong affinity for heparin.

The pattern of VEGF expression suggests its involvement in the development and maintenance of the normal vascular system and in tumor angiogenesis. During murine development, the entire 7.5 day post-coital (p.c.) endoderm expresses VEGF and the ventricular neuroectoderm produces VEGF at the capillary ingrowth stage. Breier, et al., *Development*, 114:521-523 (1992). On day two of quail development, the vascularized area of the yolk sac as well as the whole embryo show expression of VEGF. In addition, epithelial cells next to fenestrated endothelia in adult mice show persistent VEGF expression, suggesting a role in the maintenance of this specific endothelial phenotype and function.

Two high affinity receptors for VEGF have been characterized. These are VEGFR-1/Flt-1 (fms-like tyrosine kinase-1) and VEGFR-2/Kdr/Flk-1 (kinase insert domain containing receptor/fetal liver kinase-1). Those receptors are classified in the PDGF-receptor family, but they have seven rather than five immunoglobulin-like loops in their extracellular domain and they possess a longer kinase insert than normally observed in this family. The expression of VEGF receptors occurs mainly in vascular endothelial cells, although some may be present on monocytes and melanoma cells. Only endothelial cells have been reported to proliferate in response to VEGF and endothelial cells from different sources show different responses. Thus, the signals mediated through VEGFR-1 and VEGFR-2 appear to be cell type specific.

The Flt4 receptor tyrosine kinase is closely related in structure to the products of the VEGFR-1 and VEGFR-2

genes. Despite this similarity, the mature form of Flt4 differs from the VEGF receptors in that it is proteolytically cleaved in the extracellular domain into two disulfide-linked polypeptides. Pajusola et al., *Cancer Res.*, 52:5738-5743 (1992). The 4.5 and 5.8 kb Flt4 mRNAs encode polypeptides which differ in their C-termini due to the use of alternative 3' exons. The VEGFs do not show specific binding to Flt4 or induce its autophosphorylation.

Expression of Flt4 appears to be more restricted than expression of VEGFR-1 or VEGFR-2. The expression of Flt4 first becomes detectable by in situ hybridization in the angioblasts of head mesenchyme, the cardinal vein, and extraembryonically in the allantois of 8.5 day p.c. mouse embryos. In 12.5 day p.c. embryos the Flt4 signal is observed in developing venous and presumptive lymphatic endothelia, but arterial endothelia appear negative. During later stages of development, Flt4 mRNA becomes restricted to developing lymphatic vessels. Only the lymphatic endothelia and some high-endothelial venules express Flt4 mRNA in adult human tissues and increased expression occurs in lymphatic sinuses in metastatic lymph nodes and in lymphangioma. These results support the theory of the venous origin of lymphatic vessels.

#### SUMMARY OF THE INVENTION

The present invention provides a ligand for the Flt4 receptor tyrosine kinase. In a preferred embodiment, the ligand comprises a fragment of the amino acid sequence shown in SEQ ID NO: 33 which specifically binds to the Flt4 receptor tyrosine kinase.

The present invention also provides a precursor of an Flt4 ligand, wherein the precursor comprises the amino acid sequence shown in SEQ ID NO: 33. The precursor is proteolytically cleaved upon expression to produce an approximately 23 kD peptide which is the Flt4 ligand. In a preferred embodiment of the invention, an Flt4 ligand is provided which is the cleavage product of the precursor peptide shown in SEQ ID NO: 33 and which has a molecular weight of approximately 23 kD under reducing conditions. The Flt4 ligand comprises approximately the first 180 amino acids shown in SEQ ID NO: 33.

Also in a preferred embodiment, nucleic acids encoding an Flt4 ligand precursor are presented. Due to the degeneracy of the genetic code, numerous such coding sequences are possible, each having in common the coding of the amino acid sequence shown in SEQ ID NO: 33, or portions thereof. Ligand precursors according to the invention, when expressed in an appropriate host cell, produce, via cleavage, a peptide which binds specifically to the Flt4 receptor tyrosine kinase. The nucleotide sequence encoding the Flt4 ligand is within the nucleotide sequence shown in SEQ ID NO: 32.

The present invention also provides a cell line which produces an Flt4 ligand. The ligand may be purified and isolated directly from the cell culture medium. Also provided are vectors comprising DNA encoding the Flt4 ligand and host cells comprising the vectors. Vectors are capable of expressing the Flt4 ligand under the control of appropriate promoters and other control sequences.

Ligands according to the invention may be labeled with a detectable label and used to identify their corresponding receptors in situ. Antibodies, both monoclonal and polyclonal, may be made against a ligand of the invention according to standard techniques in the art. Such antibodies may be used in diagnostic applications to monitor angiogenesis, vascularization, lymphatic vessels and their

disease states, wound healing, or certain hematopoietic or leukemia cells or they may be used to block or activate the Flt4 receptor. Labeled Flt4 ligand and anti-Flt4 ligand antibodies may be used as imaging agents in the detection of lymphatic vessels, high endothelial venules, and Flt4 receptors expressed in histochemical tissue sections. The ligand or antibody may be covalently or non-covalently coupled to a suitable supermagnetic, paramagnetic, electron dense, echogenic, or radioactive agent for imaging. Other, non-radioactive labels, such as biotin and avidin may also be used.

The present invention also provides diagnostic and clinical applications for claimed ligands. In a preferred embodiment, Flt4 ligands or precursors of the invention are used to accelerate angiogenesis e.g. during wound healing or to promote the endothelial functions of lymphatic vessels. Ligands may be applied in any suitable manner using an appropriate pharmaceutically-acceptable vehicle. Ligands may also be used to quantify future metastatic risk by assaying biopsy material for the presence of active receptors or ligands in a binding assay or kit using detectably-labeled ligand. An Flt4 ligand according to the invention may also be used to promote re-growth or permeability of lymphatic vessels in, for example, organ transplant patients. Ligands according to the invention may also be used to treat or prevent inflammation, edema, aplasia of the lymphatic vessels, lymphatic obstruction, elephantiasis, and Milroy's disease. Finally, Flt4 ligands may be used to stimulate lymphocyte production and maturation and to promote or inhibit trafficking of leukocytes between tissues and lymphatic vessels or to affect migration in and out of the thymus.

Inhibitors of the Flt4 ligand may be used to control endothelial cell proliferation and lymphangiomas. For example, such inhibitors may be used to arrest metastatic growth or spread or to control other aspects of endothelial cell expression and growth. Inhibitors include antibodies, antisense oligonucleotides, and peptides which block the Flt4 receptor.

#### DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic diagram showing major endothelial cell receptor tyrosine kinases and growth factors involved in vasculogenesis and angiogenesis.

FIGS. 2A and 2B show schematically the construction of the pLTRFlt4 expression vector.

FIG. 3 shows schematically the construction of the baculovirus vector encoding a secreted soluble Flt4EC domain.

FIG. 4 shows results of stimulation of Flt4 autophosphorylation by conditioned medium from PC-3 cell cultures.

FIGS. 5A-5C show that the tyrosyl phosphorylated polypeptide of Flt4-transfected cells stimulated with PC-3 conditioned medium is the 125 kD Flt4 polypeptide.

FIGS. 6A and 6B show Western analysis of the Flt4 ligand activity isolated from PC-3 conditioned medium.

FIG. 7 shows results of gel electrophoresis of fractions from the Western analysis of Flt4 ligand isolated from PC-3 conditioned medium.

FIG. 8 shows results of Western analysis of Flt4 autophosphorylation induced by either the Flt4 ligand, VEGF, or PlGF.

FIGS. 9A through 9C show the nucleotide and deduced amino acid sequence of the coding portion of Flt4 ligand cDNA.

FIGS. 10A and 10B show a comparison of the deduced amino acid sequences of PDGF-A, -B, two PlGF isoforms, four VEGF isoforms and Flt4 ligand.

FIG. 11 shows the stimulation of autophosphorylation of the Flt4 receptor by conditioned medium from cells transfected with the Flt4-L expression vector

FIG. 12 shows Northern blotting analysis of Flt4-L mRNA in tumor cell lines.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to novel growth factors which are ligands for the Flt4 receptor tyrosine kinase. Claimed ligands are members of a family of platelet-derived growth factors/vascular endothelial growth factors which promote mitosis and proliferation of vascular endothelial cells and/or mesodermal cells. Ligands recognizing the Flt4 receptor tyrosine kinase were purified from a PC-3 prostatic adenocarcinoma cell line (ATCC CRL1435). When applied to a population of cells expressing the Flt4 receptor, ligands of the invention stimulate autophosphorylation, resulting in receptor activation. The invention also provides inhibitors of the Flt4 receptor, including antibodies directed against the receptor. A ligand according to the invention may be coexpressed as a larger precursor which is cleaved to produce the ligand. A coexpressed region in some cases results from alternative splicing of RNA of the ligand gene. Such a co-expressed region may be a function of the particular expression system used to obtain the ligand. The skilled artisan understands that in recombinant production of proteins, additional sequence may be expressed along with a functional peptide depending upon the particular recombinant construct used to express the protein, and subsequently removed to obtain the desired ligand. In some cases the recombinant ligand can be made lacking certain residues of the endogenous/natural ligand. Moreover, it is well known in that conservative replacements may be made in a protein which do not alter the function of the protein. Accordingly, it is anticipated that such alterations are within the scope of the claims. It is intended that the precursor sequence shown in SEQ ID NO: 33 is capable of stimulating the Flt4 ligand without any further processing in a manner similar to that in which VEGF stimulates its receptor in its unprocessed form.

The following Examples illustrate preferred embodiments of the invention, wherein the isolation, characterization, and function of Flt4 ligands according to the invention is shown.

#### EXAMPLE 1

##### Production of pLTRFlt4L Expression Vector

Construction of the LTR-Flt4L vector is schematically shown in FIGS. 2A and 2B. The full-length Flt4s cDNA (Genbank Accession No: X68203) was assembled by first subcloning the S2.5 fragment, reported in Pajusola et al., *Cancer Res.* 52:5738-5743 (1992), incorporated by reference herein, containing base pairs 56-2534 of the Flt4s into the EcoRI site of the pSP73 vector (Promega, Madison, Wis.).

Since cDNA libraries used for screening of Flt4 cDNAs did not contain its most 5' protein-coding sequences, inverse PCR was used for the amplification of the 5' end of Flt4 corresponding to the first 12 amino acid residues (MQRGAALCLRLW). PolyA+RNA was isolated from the HEL cells and double-stranded cDNA copy was synthesized using the Amersham cDNA Synthesis System Plus kit and a gene specific primer: 5'-TGTCTCGCTGTCCTTGCT-3' (SEQ ID NO: 1), which was located 195 bp downstream of the 5' end of clone S2.5. Double stranded cDNA was treated

with T4 DNA polymerase to blunt the ends and cDNA was purified with Centricon 100 (Amicon Inc., Beverly, Mass.). Circularization was made in a total volume of 150  $\mu$ l. The reaction mixture contained ligation buffer, 5% PEG-3000, 1 mM DTT and 8 U of T4 DNA ligase (New England Biolabs). Ligation was carried out at 16° C. for 16 hours. Fifteen  $\mu$ l of this reaction mix was used in a standard 100  $\mu$ l PCR reaction containing 100 ng of specific primers including SacI and PstI restriction sites, present in this segment of the Flt4 cDNA, and 1 unit of Taq DNA polymerase (Perkin Elmer Cetus). Two rounds of PCR were performed using 33 cycles (denaturation at 95° C. for 1 minute, annealing at 55° C. for 2 minutes and elongation at 72° C. for 4 minutes). The PCR mixture was treated sequentially with the SacI and PstI restriction enzymes and after purification with MagicPCR Preps (Promega) DNA fragments were subcloned into the pGEM3Zf(+) vector for sequencing. The sequence obtained corresponds to the 5' end of the Flt4s cDNA clone deposited in the Genbank Database as Accession No. X68203.

The sequence encoding the first 12 amino acid residues was added to the expression construct by ligating an SphI digested PCR fragment amplified using reverse transcription-PCR of polyA+RNA isolated from the HEL cells using the oligonucleotides 5'-ACATGCATGC CAC-CATGCAG.CGGGGCGCCG CGCTGTGCCT GCGACTGTGG CTCTGCCTGG GACTCCTGGA-3' (SEQ ID NO: 2) (forward primer, SphI site underlined, the translational start codon marked in bold follows an optimized Kozak consensus sequence Kozak, *Nucl. Acids Res.* 15: 8125-8148, 1987) and 5'-ACATGCATGC CCCGCCGGT CATCC-3' (SEQ ID NO: 3) (reverse primer, SphI site underlined) to the 5' end of the S2.5 fragment, thus replacing unique SphI fragment of the S2.5 plasmid. The resulting vector was digested with EcoRI and ClaI and ligated to a 138 bp PCR fragment amplified from the 0.6 kb EcoRI fragment (base pairs 3789 to 4416 in the Genbank X68203 sequence) which encodes the 3' end of Flt4s shown in FIG. 1 of Pajusola et al., *Cancer Res.* 52:5738-5743, 1992, using the oligonucleotides 5'-CGGAATTCCC CATGACCCCAAC-3' (SEQ ID NO: 4) (forward, EcoRI site underlined) and 5'-CC ATCGATGG ATCCTACCTG AAGCCGCTTT CTT-3' (SEQ ID NO: 5) (reverse, ClaI site underlined). The coding domain was completed by ligation of the 1.2 kb EcoRI fragment (base pairs 2535-3789 of sequence X68203) into the above construct. The complete cDNA was subcloned as a HindIII-ClaI(blunted) fragment (this ClaI site was also included in the 3' primer used to construct the 3' end of the coding sequence) to the pLTRpoly expression vector reported in Mäkelä et al., *Gène*, 118: 293-294 (1992) (Genbank accession number X60280), incorporated by reference herein, using its HindIII-Acc I(blunted) restriction sites.

The long form of Flt4 was produced by replacing the 3'-end of the short form as follows: The 3' region of the Flt4L cDNA was PCR-amplified using a gene specific and a pGEM 3Z vector specific (SP6 promoter) oligonucleotide 5'-ATTTAGGTGACACTATA-3' (SEQ ID NO: 6) as reverse and forward primers, respectively, and an Flt4L cDNA clone containing a 495 bp EcoRI fragment extending downstream of the EcoRI site at nucleotide 3789 of the Genbank X68203 sequence (the sequence downstream of this EcoRI site is deposited as the Flt4 long form 3' sequence having Genbank accession number S66407). The gene specific oligonucleotide contained a BamHI restriction site located right after the end of the coding region. The sequence of that (reverse primer) oligonucleotide was 5'-CCATCGAT GGATCCCGATGCTGCTTAGTAGCTGT-3' (SEQ ID NO:



7) (BamHI site is underlined). The PCR product was digested with EcoRI and BamHI and transferred in frame to LTRFlt4s vector fragment from which the coding sequences downstream of the EcoRI site at base pair 2535 (see sequence X68203) had been removed by EcoRI-BamHI digestion. Again, the coding domain was completed by ligation of the 1.2 kb EcoRI fragment (base pairs 2535-3789 of sequence X68203) back into the resulting construct.

#### EXAMPLE 2

##### Production and Analysis of Flt4l Transfected Cells

NIH3T3 cells (60% confluent) were cotransfected with 5 µg of the pLTRFlt4l construct and 0.25 µg of the pSV2neo vector (ATCC) containing the neomycin phosphotransferase gene, using the DOTAP liposome-based transfection reagents (Boehringer Mannheim, Mannheim, Germany). One day after the transfection the cells were transferred into selection media containing 0.5 mg/ml geneticin (GIBCO, Grand Island, N.Y.). Colonies of geneticin-resistant cells were isolated and analysed for expression of the Flt4 proteins. Cells were lysed in boiling lysis buffer containing 3.3% SDS (sodium dodecyl sulphate), 125 mM Tris, pH 6.8. Protein concentrations of the samples were measured by the BCA method (Pierce, Rockford, Ill.). About 50 µg protein of each lysate was analysed for the presence of Flt4 by 6% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting using antisera against the carboxyl terminus of Flt4 and the ECL method (Amersham).

For production of anti-Flt4 antiserum the Flt4 cDNA fragment encoding the 40 carboxyterminal amino acid residues of the short form: NH<sub>2</sub>-PMTPTTYKG SVDN-QTDSGM VLASEEFEQI ESRHRQESGFR-COOH (SEQ ID NO: 8) was cloned as a 657 bp EcoRI-fragment into the pGEX-11T bacterial expression vector (Pharmacia) in frame with the glutathione-S-transferase coding region. The resulting GST-Flt4S fusion protein was produced in *E. coli* and purified by affinity chromatography using a glutathione-Sepharose 4B column. The purified protein was lyophilized, dissolved in phosphate buffered saline (PBS), mixed with Freund's adjuvant and used for immunization of rabbits at biweekly intervals using methods standard in the art (Harlow and Lane, Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1988). Antisera were used after the fourth booster immunization for immunoprecipitation of Flt4 from the transfected cells and clones expressing Flt4 were used for ligand stimulation analysis.

#### EXAMPLE 3

##### Construction of a Flt4 EC Baculovirus Vector and Expression and Purification of its Product

The construction of an Flt4 extracellular domain (EC) baculovirus vector is schematically shown in FIG. 3. The Flt4-encoding cDNA has been prepared in both a long form and a short form, each being incorporated in a vector under control of the Moloney murine leukemia virus LTR promoter. The nucleotide sequence of the short form of the Flt4 receptor is available on the Genbank database as Accession No. X68203 and the specific 3' segment of the long form cDNA is available as Accession No. S66407.

The ends of a cDNA segment encoding Flt4 extracellular domain (EC) were modified as follows: The 3' end of Flt4 cDNA sequence (Genbank Accession Number X68203) which encodes the extracellular domain was amplified using primer 1116 5'-CTGGAGTCTGACTTGGCGGACT-3' (SEQ

ID NO: 9, SalI site underlined) and primer 1315 5'-CGC GGATCCCTAGTGATGGTGATGGTGATGTCTACCTTC GATCATG CTGCCCTTAT CCTC-3' (SEQ ID NO: 10, BamHI site underlined). The sequence complementary to that of primer 1315 continues after the Flt4 reading frame and encodes 6 histidine residues for binding to a Ni-NTA column (Qiagen, Hilden, Germany) followed by a stop codon, and an added Bam HI site. The amplified fragment was digested with SalI and BamHI and used to replace a unique SalI-BamHI fragment in the LTRFlt4 vector shown in FIG. 3. The SalI-BamHI fragment that was replaced encodes the Flt4 transmembrane and cytoplasmic domains.

The 5' end without the Flt4 signal sequence encoding region was amplified by PCR using the primer 1335 5'-CCC AAGCTTGGATCCAAGTGGCTACTCCATGACC-3' (SEQ ID NO: 11) (the primer contains added HindIII (AAGCTT) and BamHI (GGATCC) restriction sites, which are underlined) and primer 1332 5'-GTTGCCTGTGATGTGCACCA-3' (SEQ ID NO: 12). The amplified fragment was digested with HindIII and SphI (the HindIII site (AAGCTT) is underlined in primer 1335 and the SphI site is within the amplified region of the Flt4 cDNA). The resulting HindIII-SphI fragment was used to replace a HindIII-SphI fragment in the modified LTRFlt4 vector described immediately above (the HindIII site is in the 5' junction of the Flt4 insert with the pLTRpoly portion of the vector, the SphI site is in Flt4 cDNA). The resulting Flt4EC insert was then ligated as a BamHI fragment into the BamHI site in the pVTBac plasmid as disclosed in Tessier et al., *Gene* 98: 177-183 (1991), incorporated by reference herein. The orientation was confirmed to be correct by partial sequencing so that the open reading frame of the signal sequence-encoding portion of the vector continued in frame with the Flt4 sequence. That construct was transfected together with the baculovirus genomic DNA into SF-9 cells by lipofection. Recombinant virus was purified, amplified and used for infection of High-Five cells (Invitrogen, San Diego, Calif.) using methods standard in the art. The Flt4 extracellular domain was purified from the culture medium of the infected High-Five cells using Ni-NTA affinity chromatography according to manufacturer's instructions (Qiagen) for binding and elution of the 6xHis tag encoded in the COOH-terminus of the recombinant Flt4 extracellular domain.

#### EXAMPLE 4

##### Isolation of Flt4 Ligand from Conditioned Media

An Flt4 ligand according to the invention was isolated from conditioned media from PC-3 prostatic adenocarcinoma cell line CRL1435 from the American Type Culture Collection and cultured as instructed by the supplier in Ham's F-12 Nutrient mixture (GIBCO) containing 7% fetal calf serum. In order to prepare the conditioned media, confluent PC-3 cells were cultured for 7 days in Ham's F-12 Nutrient mixture (GIBCO) in the absence of fetal bovine serum. Medium was then cleared by centrifugation at 10,000 g for 20 minutes. The medium was then screened to determine its ability to induce tyrosine phosphorylation of Flt4 by exposure to NIH3T3 cells which had been transfected with Flt4-encoding cDNA using the pLTRFlt4l vector. For receptor stimulation experiments, subconfluent NIH3T3 cells were starved overnight in serum-free DMEM medium (GIBCO) containing 0.2% BSA. The cells were stimulated with the conditioned media for 5 minutes, washed twice with cold PBS containing 100 µM vanadate and lysed in RIPA buffer (10 mM Tris pH 7.5, 50 mM NaCl, 0.5% sodium



deoxycholate, 0.5% Nonidet P40 (BDH, Poole, England), 0.1% SDS, 0.1 U/ml Aprotinin (Boehringer Mannheim), 100  $\mu$ M vanadate) for receptor immunoprecipitation analysis. The lysates were centrifuged for 20 minutes at 15,000 $\times$ g. The supernatants were incubated for 2 hours on ice with 3  $\mu$ l of the antiserum against the Flt4 C-terminus described in Example 2 and also in Pajusola, et al. *Oncogene* 8: 2931-2937, (1993), incorporated by reference herein.

After a 2 hour incubation in the presence of anti-Flt4 antiserum, protein A-Sepharose (Pharmacia) was added and incubation was continued for 45 minutes with rotation. The immunoprecipitates were washed three times with the immunoprecipitation buffer and twice with 10 mM Tris, pH 7.5 before analysis in SDS-PAGE. Polypeptides were transferred to nitrocellulose and analyzed by Western blotting using Flt4- or phosphotyrosine-specific antisera and the ECL method (Amersham International, Buckinghamshire, England). Anti-phosphotyrosine monoclonal antibodies (anti-PTyr; PY20) were purchased from Transduction Laboratories (Lexington, Ky.). In some cases, the filters were restained with a second antibody after stripping. The stripping of the filters was done for 30 minutes at 50 $^{\circ}$  C. in 100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7 with occasional agitation.

As shown in FIG. 4, the PC-3 cell conditioned medium stimulated tyrosine phosphorylation of a 125 kD polypeptide when Flt4 expressing NIH3T3 cells were treated with the indicated preparations of media, lysed, and the lysates were immunoprecipitated with anti-Flt4 antiserum followed by SDS-PAGE, Western blotting, and staining using anti-PTyr antibodies. The resulting band was weakly phosphorylated upon stimulation with unconcentrated PC-3 conditioned medium (lane 2). The 125 kD band comigrated with the tyrosine phosphorylated, processed form of the mature Flt4 from pervanadate-treated cells (compare lanes 2 and 7 of FIG. 4, see also FIG. 5A). Comigration was confirmed upon restaining with anti-Flt4 antibodies as is also shown in FIG. 5A (panel on the right). In order to show that the 125 kD polypeptide is not a non-specific component of the conditioned medium reactive with anti-phosphotyrosine antibodies, 15  $\mu$ l of conditioned medium was separated by SDS-PAGE, blotted on nitrocellulose and the blot was stained with anti-PTyr antibodies. No signal was obtained (FIG. 5B). Also, unconditioned medium failed to stimulate Flt4 phosphorylation, as shown in FIG. 4, lane 1.

As shown in FIG. 4, lane 3, stimulating activity was considerably increased when the PC-3 conditioned medium was concentrated four-fold using a Centricon-10 concentrator (Amicon). FIG. 4, lane 4, shows that pretreatment of the concentrated PC-3 conditioned medium with 50  $\mu$ l of the Flt4 extracellular domain coupled to CNBr-activated sepharose CL-4B (Pharmacia; about 1 mg of Flt4 EC domain/ml sepharose resin) completely abolished Flt4 tyrosine phosphorylation. Similar pretreatment of the conditioned medium with unsubstituted sepharose CL-4B did not affect stimulatory activity, as shown in FIG. 4, lane 5. Also, the flow through obtained after concentration, which contained proteins of less than 10,000 molecular weight, did not stimulate Flt4 phosphorylation, as shown in FIG. 4, lane 6.

The foregoing data show that PC-3 cells produce a ligand which binds to the extracellular domain of Flt4 and activates this receptor.

#### EXAMPLE 5

##### Purification of the Flt4 Ligand

The ligand expressed by PC-3 cells as characterized in Example 3 was purified and isolated using a recombinant by produced Flt4 extracellular domain in affinity chromatography.

Two harvests of serum-free conditioned medium, comprising a total of 8 L were collected from 500 confluent 15 cm diameter culture dishes containing confluent layers of PC-3 cells. The conditioned medium was clarified by centrifugation at 10,000 $\times$ g and concentrated 80-fold using an Ultrasette Tangential Flow Device (Filtron, Northborough, Mass.) with a 10 kD cutoff Omega Ultrafiltration membrane according to the manufacturer's instruction. Recombinant Flt4 extracellular domain was expressed in a recombinant baculovirus cell system and purified by affinity chromatography on Ni-agarose (Ni-NTA affinity column obtained from Qiagen). The purified extracellular domain was coupled to CNBr-activated Sepharose CL-4B at a concentration of 5 mg/ml and used as an affinity matrix for ligand affinity chromatography.

Concentrated conditioned medium was incubated with 2 ml of the recombinant Flt4 extracellular domain-Sepharose affinity matrix in a rolling tube at room temperature for 3 hours. All subsequent purification steps were at +4 $^{\circ}$  C. The affinity matrix was then transferred to a column (Pharmacia) with an inner diameter of 15 mm and washed successively with 100 ml of PBS and 50 ml of 10 mM Na-phosphate buffer (pH 6.8). Bound material was eluted step-wise with 100 mM glycine-HCl, successive 5 ml elutions having pHs of 4.0, 2.4, and 1.9. Several 2 ml fractions of the eluate were collected in tubes containing 0.5 ml 1 M Na-phosphate (pH 8.0). Fractions were mixed immediately and dialysed in 1 mM Tris-HCl (pH 7.5). Aliquots of 75  $\mu$ l each were analyzed for their ability to stimulate tyrosine phosphorylation of Flt4. The ultrafiltrate, 100  $\mu$ l aliquots of the concentrated conditioned medium before and after ligand affinity chromatography, as well as 15-fold concentrated fractions of material released from the Flt4 extracellular domain-Sepharose matrix during the washings were also analyzed for their ability to stimulate Flt4 tyrosine phosphorylation.

As shown in FIGS. 6A and 6B, lane 3, the concentrated conditioned medium induced prominent tyrosine phosphorylation of Flt4 in transfected NIH3T3 cells overexpressing Flt4. This activity was not observed in conditioned medium taken after medium was exposed to the Flt4-Sepharose affinity matrix described above (lane 4). The specifically-bound Flt4-stimulating material was retained on the affinity matrix upon washes in PBS, 10 mM Na-phosphate buffer (pH 6.8), and at pH 4.0 (lanes 5-7, respectively), and it was eluted in the first two 2 ml aliquots at pH 2.4 (lanes 8 and 9). A further decrease of the pH of the elution buffer did not cause release of additional Flt4-stimulating material (lane 11).

Small aliquots of the chromatographic fractions were concentrated in a SpeedVac concentrator (Savant, Farmingdale, N.Y.) and subjected to SDS-PAGE under reducing conditions with subsequent silver staining of the gel. As shown in FIG. 7, the major polypeptide, having a molecular weight of approximately 23 kD (reducing conditions), was detected in the fractions containing Flt4 stimulating activity (corresponding to lanes 8 and 9 in FIG. 6). That polypeptide was not found in the other chromatographic fractions. On the other hand, all other components detected in the two active fractions were also distributed in the starting material and in small amounts in the other washing and elution steps after their concentration. Similar results were obtained in three independent affinity purifications, indicating that the 23 kD polypeptide specifically binds to Flt4 and induces its tyrosine phosphorylation.

Fractions containing the 23 kD polypeptide were combined, dried in a SpeedVac concentrator and subjected to SDS-PAGE in a 12.5% gel. The proteins from the gel

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were then electroblotted to Immobilon-P (PVDF) transfer membrane (Millipore, Marlborough, Mass.) and visualized by staining of the blot with Coomassie blue R-250. The region containing only the stained 23 kD band was cut from the blot and was subjected to N-terminal amino acid sequence analysis in a Prosite Protein Sequencing System (Applied Biosystems, Foster City, Calif.). The data were analyzed using a 610A Data Analysis System (Applied Biosystems). Analysis revealed a single N-terminal sequence of NH<sub>2</sub>-XEETIKFAAAHYNTEILK-COOH (SEQ ID NO: 13).

## EXAMPLE 6

## Construction of PC-3 Cell cDNA Library in a Eukaryotic Expression Vector

Poly-A+RNA was isolated from five 15-cm diameter confluent dishes of PC-3 cells by a single step method using oligo(dT) (Type III, Collaborative Research) cellulose affinity chromatography (Sambrook et al., Molecular Cloning, A Laboratory Manual; Cold Spring Harbor Laboratory Press, 1989). The yield was 70 µg. Six µg of the poly-A+RNA was used to prepare an oligo(dT)-primed cDNA library in the mammalian expression vector pcDNA1 and the Librarian kit of Invitrogen according to the instructions included in the kit. The library was estimated to contain about 10<sup>6</sup> independent recombinants with an average insert size of approximately 1.8 kb.

## EXAMPLE 7

## Amplification of the Unique Nucleotide Sequence Encoding the Flt4 Ligand

Degenerate oligonucleotides were designed based on the N-terminal amino acid sequence of the isolated Flt4 ligand and were used as primers in a polymerase chain reaction (PCR) to amplify cDNA encoding the Flt4 ligand from a PC-3 cell library.

The PCR was carried out using 1 µg of DNA from the amplified PC-3 cDNA library and a mixture of sense-strand primers comprising 5'-GCAGARGARACNATHAA-3' (SEQ ID NO: 14) (wherein R is A or G, N is A, G, C or T and H is A, C or T), encoding amino acid residues 2-6 (EETIK, SEQ ID NO: 15) and antisense-strand primers 5'-GCAYTTNARDATYTCNGT-3' (SEQ ID NO: 16) (wherein Y is C or T and D is A, G or T), corresponding to amino acid residues 14-18 (TEILK, SEQ ID NO: 17). Three extra nucleotides (GCA) were added to the 5'-terminus of each primer to increase annealing stability. Two successive PCR runs were carried out using 1 U per reaction of DynaZyme, a thermostable DNA polymerase (F-500L, Finnzymes), in a buffer supplied by the manufacturer (10 mM Tris-HCl, pH 8.8 at 25° C., 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.1% Triton-X100) at an extension temperature of 72° C. The first PCR run was carried out for 43 cycles. The first three cycles were run at annealing temperature 33° C. for 2 minutes and the remaining cycles were run at 42° C. for 1 minute.

The region of the gel containing a weak band of the expected size (57 bp) was cut out from the gel and eluted. The eluted material was reamplified for 30 cycles using the same primer pairs described above at 42° C. for 1 minute. The amplified fragment was cloned into a PCR II vector (Invitrogen) using the TA cloning kit (Invitrogen) and sequenced using the radioactive dideoxynucleotide sequencing method of Sanger. Six clones were analysed and all

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contained the sequence encoding the expected peptide (amino acids 2-18 of the Flt4 ligand precursor). Nucleotide sequence spanning the region from the third nucleotide of codon 6 to the third nucleotide of codon 13 (the extension region) was identical in all six clones: 5'-ATTGCTGCAGCACTACAAC-3' (SEQ ID NO: 18) and thus was considered to represent an amplified product from the unique sequence encoding part of the amino terminus of the Flt4 ligand.

## EXAMPLE 8

## Amplification of the 5'-end of the cDNA Encoding the Flt4 Ligand

Based on the unique nucleotide sequence encoding the N-terminus of the isolated Flt4 ligand, two pairs of nested primers were designed to amplify in two subsequent PCR-reactions the complete 5'-end of the corresponding cDNAs from 1 µg of DNA from the above-described PC-3 cDNA library. First, amplification was performed with primer 5'-TCNGTGTGTAGTGTGCTG-3' (SEQ ID NO: 19) which is the antisense-strand primer corresponding to amino acid residues 9-15 (AAHYNTE, SEQ ID NO: 20) and sense-strand primer 5'-TAATACGACTCACTATAGGG-3' (SEQ ID NO: 21), corresponding to the T7 RNA promoter of the pcDNA1 vector used for construction of the library. "Touchdown" PCR was used as disclosed in Don, et al., *Nucl. Acids Res.*, 19: 4008 (1991), incorporated by reference herein. The annealing temperature of the two first cycles was 62° C. and subsequently the annealing temperature was decreased in every other cycle by 1° C. until a final temperature of 53° C. was reached, at which temperature 16 additional cycles were carried out. Annealing time as 1 minute and extension at each cycle was conducted at 72° C. for 1 minute. Multiple amplified DNA fragments were obtained in the first reaction. The products of the first amplification (1 µl of a 1:100 dilution in water) were used in the second amplification reaction employing the nested primers 5'-GTTGTAGTGTGCTGCAGCGAATTT-3' (SEQ ID NO: 22), an antisense-strand primer corresponding to amino acid residues 6-13 (KFAAAHYN, SEQ ID NO: 23) of the Flt4 ligand and 5'-TCACTATAGGGAGACCCAAGC-3' (SEQ ID NO: 24), a sense-strand primer corresponding to nucleotides 2179-2199 of the pcDNA1 vector. The sequences of these sense and antisense primers overlapped with the 3' ends of the corresponding primers used in the first PCR. "Touchdown" PCR was carried out by decreasing the annealing temperature from 72° C. to 66° C. and continuing with 18 additional cycles at 66° C. The annealing time was 1 minute and extension at each cycle was carried out at 72° C. for 2 minutes. One major product of about 220 bp and three minor products of about 270 bp, 150 bp, and 100 bp were obtained.

The amplified fragment of approximately 220 bp was cut out from the agarose gel, cloned into a pCRII vector using the TA cloning kit (Invitrogen) and sequenced. Three recombinant clones were analysed and they contained the sequence  
5'-TCACTATAGGGAGACCCAAGCTTGGTACCGAGCTCGGATCCACTA GTAACGGCCGCCAGTGTGGTGCAATTCGACGAATCATGACTGTA  
CTCTACCCAGAATATTGGAAAATGTACAAAGTGTCAGCTAAGGCAA  
GTAGGCTGGCAACATAACAGAGAACAGGCCAACC  
TCAACTCAAG  
GACAGAAGAGACTATAAAATTCGCTGCAGCACTACAAC-3' (SEQ ID NO: 25). The beginning of the

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sequence represents the pcDNA1 vector and the underlined sequence represents the amplified product of the 5'-end of the insert. The ATG codon located upstream of that sequence in the same reading frame is followed by an open reading frame containing the amplified product of the putative signal sequence and the first 13 amino acid residues of the secreted Flt4 ligand.

## EXAMPLE 9

## Amplification of the 3'-end of cDNA Encoding the Flt4 Ligand

Based upon the amplified 5'-sequence of the clones encoding the Flt4 ligand, two pairs of non-overlapping, nested primers were designed to amplify the 3'-portion of the clones. The sense-strand primer 5'-ACAGAGAACAGGCCAACC-3' (SEQ ID NO: 26) and antisense-strand primer 5'-TCTAGCATTAGGTGACAC-3' (SEQ ID NO: 27) corresponding to nucleotides 2311-2329 of the pcDNA1 vector were used in a first "touchdown" PCR. The annealing temperature of the reaction was decreased 1° C. every two cycles from 72° C. to 52° C., at which temperature 15 additional cycles were carried out. The annealing time was 1 minute and extension at each cycle was carried out at 72° C. for 3 minutes. DNA fragments of several sizes were obtained in the first amplification. Those products were diluted 1:200 in water and reamplified in PCR using the second pair of primers: 5'-AAGAGACTATAAAATTCGCTGCAGC-3' (SEQ ID NO: 28) and 5'-CCCTCTAGATGCATGCTCGA-3' (SEQ ID NO: 29) (antisense-strand primer corresponding to nucleotides 2279-2298 of the pcDNA1 vector). Two DNA fragments were obtained, having sizes of 1350 bp and 570 bp. Those fragments were cloned into a pCRII vector and the inserts of the clones were sequenced. Both of these fragments were found to contain sequences encoding an amino acid sequence homologous to the VEGF sequence.

## EXAMPLE 10

## Screening the PC-3 Cell cDNA Library using the 5' PCR Fragment of Flt4 Ligand cDNA

A 219 bp 5'-terminal fragment of Flt4 ligand cDNA was amplified by PCR using the 5' PCR fragment described above and primers 5'-GTTGTAGTGTGCTGCAGCGAATTT-3' (antisense-strand primer, SEQ ID NO: 30) and 5'-TCACTATAGGGAGACCCAAGC-3' (SEQ ID NO: 31) (sense-primer corresponding to nucleotides 2179-2199 of the pcDNA1 vector). The amplified product was subjected to digestion with EcoRI (Boehringer Mannheim) to remove the portion of the DNA sequence amplified from the pcDNA1 vector and the resulting 153 bp fragment encoding the 5' end of the Flt4 ligand was labeled with [<sup>32</sup>P]-dCTP using the Klenow fragment of *E. coli* DNA polymerase I (Boehringer Mannheim). That fragment was used as a probe for hybridization screening of the amplified PC-3 cell cDNA library.

Filter replicas of the library were hybridized with the radioactively labeled probe at 42° C. for 20 hours in a solution containing 50% formamide, 5×SSPE, 5×Denhardt's solution, 0.1% SDS and 0.1 mg/ml denatured salmon sperm DNA. Filters were washed twice in 1×SSC, 0.1% SDS for 30 minutes at room temperature, then twice for 30 minutes at 65° C. and exposed overnight.

On the basis of autoradiography, 10 positive recombinant bacterial colonies hybridizing with the probe were chosen

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from the library. Plasmid DNA was purified from these colonies and analysed by EcoRI and NotI digestion and agarose gel electrophoresis followed by ethidium bromide staining. The ten plasmid clones were divided into three groups on the basis of the presence of insert sizes of approximately 1.7, 1.9 and 2.1 kb, respectively. Inserts of plasmids from each group were sequenced using the T7 oligonucleotide as a primer and walking primers for subsequent sequencing reactions.

Sequence analysis showed that all clones contain the open reading frame encoding the NH<sub>2</sub>-terminal sequence of the Flt4 ligand. Furthermore, the 2.1 and 1.9 kb clones also contained sequences encoding the signal sequence. The 5' end of the 1.7 kb clone began within the signal sequence-encoding portion. Dideoxy sequencing was continued using walking primers in the downstream direction. An 1140 nucleotide portion of the sequence of the longest clone is shown in FIGS. 9A through 9C (SEQ ID NOS: 32 and 33). As can be seen in that figure, after the putative signal sequence the open reading frame terminates in a TAA stop codon 317 amino acid residues further downstream from the signal sequence. When compared with sequences in the GenBank Database, the predicted protein product of this reading frame was found to be homologous with the predicted amino acid sequences of the PDGF/VEGF family of growth factors, as shown in FIGS. 10A and 10B.

## EXAMPLE 11

## Stimulation of Flt4 Autophosphorylation by the Protein Product of the Flt4 Ligand Vector

The 2.1 kb insert of the Flt4-L clone in pcDNA1 vector containing the open reading frame encoding the sequence shown in FIGS. 9A through 9C (SEQ ID NO: 32) was cut out from the vector using HindIII and NotI restriction enzymes, isolated from a preparative agarose gel and ligated to the corresponding sites in the pREP7 expression vector (Invitrogen). The pREP7 vector containing the above cloned insert was transfected into 293-EBNA cells (Invitrogen) using the calcium phosphate transfection method (Sambrook et al., Molecular Cloning, A Laboratory Manual; Cold Spring Harbor Laboratory Press, 1989). About 48 hours after transfection the medium of the transfected cells was changed to DMEM medium lacking fetal calf serum and incubated for 36 h. The thus conditioned medium was then collected, centrifuged at 5000×g for 20 minutes, the supernatant was concentrated 5-fold using Centriprep 10 (Amicon) stimulate NIH3T3 cells expressing LTRFlt41, as in Example 4. The cells were lysed, immunoprecipitated using anti-Flt4 antiserum and analysed by Western blotting using anti-phosphotyrosine antibodies.

As can be seen from FIG. 11, lanes 1 and 3, the conditioned medium from two different dishes of the transfected cells stimulated Flt4 autophosphorylation in comparison with the medium from mock-transfected cells, which gave only background levels of phosphorylation of the Flt4 receptor (lane 2). When the concentrated conditioned medium was preabsorbed with 20 µl of a slur of Flt4EC domain coupled to Sepharose (see example 4), no phosphorylation was obtained (lane 4), showing that the activity responsible for Flt4 autophosphorylation was indeed the Flt4 ligand. Thus, these results demonstrate that the Flt4-L plasmid vector clone having an approximately 2.1 kb insert and containing the open reading frame shown in FIGS. 9A through 9C is expressed into a Flt4 ligand in cells transfected with the Flt4-L expression vector clone, and thus is biologically active. The sequence encoded by that open reading frame is shown in SEQ ID NO: 33. Plasmid pFLt4-L has been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md. 20852 as accession

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number 97231. A 1997 base pair nucleotide sequence and the deduced amino acid sequence of the cDNA insert of this deposited plasmid is set forth in SEQ ID NOS: 34 and 35, respectively.

However, the predicted molecular weight of the mature protein product deduced from this reading frame is 35,724 and the Flt4 ligand from PC-3 cell cultures had an approximate molecular weight of 23 kD under reducing conditions. It is thus possible that the Flt4 mRNA may be first translated into a precursor, from which the mature ligand is derived by proteolytic cleavage. The difference in the observed molecular weight of the isolated Flt4 ligand and the deduced molecular weight of the disclosed open reading frame of the Flt4 ligand sequence may then derive from sequences in the carboxyl terminal region of the latter. Also, the Flt4 ligand may be glycosylated at two putative N-linked glycosylation sites conforming to the consensus which can be identified in the deduced Flt4 ligand amino acid sequence (N-residues underlined in FIG. 10A).

The carboxyl terminal amino acid sequences, which increase the predicted molecular weight of the Flt4 ligand subunit in comparison with other ligands of this family show a pattern of spacing of cysteine residues reminiscent of the Balbiani ring protein 3 (BRP3) sequence (Dignam and Case, Gene 88, 133-140, 1990). Such a sequence may encode an independently folded domain present in a Flt4 ligand precursor and it may be involved, for example, in the regulation of secretion, solubility, stability, cell surface localization or activity of the Flt4 ligand. Interestingly, at least one cysteine motif of the BRP3 type is also found in the VEGF carboxy terminal amino acid sequences.

Thus, the Flt4 mRNA may be first translated into a precursor from the mRNA corresponding to the Flt4-L clone, from which the mature ligand is derived by proteolytic cleavage. To define the mature Flt4 ligand product, one first expresses the cDNA clone, which is deposited in the pcDNA1 expression vector, in cells, such as COS cells and use antibodies generated against Flt4-L-encoded peptides, such as amino terminal 23 amino acid peptide or bacterial Flt4 fusion proteins, such as a GST-fusion protein, to raise antibodies against the VEGF-homologous domain of Flt4 ligand. One then follows the biosynthesis and processing of the Flt4 ligand in the transfected cells by pulse-chase analysis using radioactive cysteine for labelling of the cells, immunoprecipitation and gel electrophoresis. Using antibodies against the two domains of the product of the Flt4-L clone material for radioactive or nonradioactive aminoterminal sequence analysis is isolated. The determination of the NH2-terminal sequence of the carboxyl terminal fragment allows for identification of the proteolytic processing site. This is confirmed by site-directed mutagenesis of the amino acid residues adjacent to the cleavage site, which would prevent the cleavage.

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On the other hand, the Flt4 ligand is characterized by progressive 3' deletions in the 3' coding sequences of the Flt4 ligand precursor clone, resulting in carboxy-terminal truncations of its protein product. The activities of such truncated forms are assayed by, for example, studying Flt4 autophosphorylation induced by the truncated proteins when applied to cultures of cells, such as NIH3T3 cells expressing LTRFlt4. By extrapolation from studies of the structure of the related platelet derived growth factor (PDGF, reference Heldin et al., Growth Factors 8, 245-252, 1993) one determines that the region critical for receptor activation by the Flt4 ligand is contained within its first approximately 180 amino acid residues.

On the other hand, the difference between the molecular weights of the purified ligand and the open reading frame of the Flt4 precursor clone may be due to the fact that the soluble ligand was produced from an alternatively spliced mRNA which would also be present in the PC-3 cells, from which the isolated ligand was derived. To isolate such alternative cDNA clones one uses cDNA fragments of the deposited clone and PCR primers made according to the sequence provided as well as techniques standard in the art to isolate or amplify alternative cDNAs from the PC-3 cell cDNA library. One may also amplify using reverse transcription (RT)-PCR directly from the PC-3 mRNA using the primers provided in the sequence of the Flt4-L clone. Alternative cDNAs can be sequenced from the resulting cDNA clones. One can also isolate genomic clones corresponding to the Flt4-L transcript from a human genomic DNA library using methods standard in the art and to sequence such clones or their subcloned fragments to reveal the corresponding exons. Alternative exons can then be identified by a number of methods standard in the art, such as heteroduplex analysis of cDNA and genomic DNA and they can subsequently be characterized.

## EXAMPLE 12

### Expression of the Flt4-L Gene

Expression of transcripts corresponding to the Flt4 ligand was analysed by hybridization of Northern blots containing isolated polyA+RNA from HT-1080 and PC-3 human tumor cell lines. The probe was the radioactively labelled insert of the 2.1 kb cDNA clone (specific activity  $10^8$ - $10^9$  cpm/mg of DNA). The blot was hybridized overnight at 42° C. or using 50% formamide, 5×SSPE buffer, 2% SDS, 10×Denhardt's solution, 100 mg/ml salmon sperm DNA and  $1 \times 10^6$  cpm of the labelled probe/ml. The blot was washed at room temperature for 2×30 minutes in 2×SSC containing 0.05% SDS and then for 2×20 min at 52° C. in 0.1×SSC containing 0.1% SDS. The blot was then exposed at -70° C. for three days using intensifying screens and Kodak XAR film. Both cell lines expressed an Flt4 ligand mRNA of about 2.3 kb, as well as VEGF and VEGF-B mRNAs (FIG. 12).

## SEQUENCE LISTING

### (1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 35

### (2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

-continued

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TGTCTCGCT GTCCTGTCT

20

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 70 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

ACATGCATGC CACCATGCAG CCGGGCGCCG CGCTGTCCT GCGACTGTGG CTCGCTGG

60

GACTCCTGGA

70

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

ACATGCATGC CCGCGCGGTC ATCC

24

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CGGAATTCCC CATGACCCCA AC

22

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CCATCGATGG ATCCTACCTG AAGCCGCTTT CTT

33

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid

-continued

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

ATTTAGGTGA CACTATA

17

(2) INFORMATION FOR SEQ ID NO: 7:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 14 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

CCATCGATGG ATCCCGATGC TGCTTAGTAG CTGT

34

(2) INFORMATION FOR SEQ ID NO: 8:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 40 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Pro Met Thr Pro Thr Thr Tyr Lys Gly Ser Val Asp Asn Gln Thr As  
 1 5 10 15

Ser Gly Met Val Leu Ala Ser Glu Glu Phe Glu Gln Ile Glu Ser Ar  
 20 25 30

His Arg Gln Glu Ser Gly Phe Arg  
 35 40

(2) INFORMATION FOR SEQ ID NO: 9:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 21 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CTGGAGTCGA CTGGCCGAC T

21

(2) INFORMATION FOR SEQ ID NO: 10:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 60 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CGCGGATCCC TAGTGATGGT GATGGTGATG TCTACCTTCG ATCATGCTGC CCTTATCCTC

60

(2) INFORMATION FOR SEQ ID NO: 11:

-continued

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 34 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CCCAAGCTTG GATCCAAGTG GCTACTCCAT GACC

34

(2) INFORMATION FOR SEQ ID NO: 12:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 20 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GTTGCCTGTG ATGTGCACCA

20

(2) INFORMATION FOR SEQ ID NO: 13:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 18 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

Xaa Glu Glu Thr Ile Lys Phe Ala Ala Ala His Tyr Asn Thr Glu Il  
 1 5 10 15

Leu Lys

(2) INFORMATION FOR SEQ ID NO: 14:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 17 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

GCAGARGARA CNATHAA

17

(2) INFORMATION FOR SEQ ID NO: 15:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 5 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

Glu Glu Thr Ile Lys  
 1 5

(2) INFORMATION FOR SEQ ID NO: 16:

-continued

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 18 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

GCAYTTNARD ATYTCNGT

18

(2) INFORMATION FOR SEQ ID NO: 17:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 5 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

Thr Glu Ile Leu Lys

1 5

(2) INFORMATION FOR SEQ ID NO: 18:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 22 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

ATTCGCTGCA GCACACTACA AC

22

(2) INFORMATION FOR SEQ ID NO: 19:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 19 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

TCAGTGTGTG ACTGTGCTG

19

(2) INFORMATION FOR SEQ ID NO: 20:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 7 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

Ala Ala His Tyr Asn Thr Glu

1 5

(2) INFORMATION FOR SEQ ID NO: 21:

- (i) SEQUENCE CHARACTERISTICS:



-continued

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

TAATACGACT CACTATAGG

20

(2) INFORMATION FOR SEQ ID NO: 22:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 24 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

GTTGTAGTGT GCTGCAGCGA ATTT

24

(2) INFORMATION FOR SEQ ID NO: 23:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 8 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

Lys Phe Ala Ala His Tyr Asn  
1 5

(2) INFORMATION FOR SEQ ID NO: 24:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 21 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

TCACTATAGG GAGACCCAAG C

21

(2) INFORMATION FOR SEQ ID NO: 25:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 219 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

TCACTATAGG GAGACCCAAG CTTGGTACCG AGCTCGGATC CACTAGTAAC GCGCCGCGAGT

60

GTGGTGAAT TCGACGAAT CATGACTGTA CTCTACCCAG AATATTGGAA AATGTACAA

120

TGTGAGCTAA GGCAGGAGG CTGGCAACAT AACAGAGAAC AGGCCAACCT CAACTCAAG

180

ACAGAAGAGA CTATAAAATT CGCTGCAGCA CACTACAAC

219

-continued

## (2) INFORMATION FOR SEQ ID NO: 26:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 18 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

ACAGAGAACA GGCCAACC

18

## (2) INFORMATION FOR SEQ ID NO: 27:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 19 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

TCTAGCATTG AGGTGACAC

19

## (2) INFORMATION FOR SEQ ID NO: 28:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 25 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

AAGAGACTAT AAAATTGCT GCAGC

25

## (2) INFORMATION FOR SEQ ID NO: 29:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

CCCTCTAGAT GCATGCTCGA

20

## (2) INFORMATION FOR SEQ ID NO: 30:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 24 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

GTTGTAGTGT GCTGCAGCGA ATTT

24

## (2) INFORMATION FOR SEQ ID NO: 31:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 21 base pairs

-continued

- (B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

TCACTATAGG GAGACCCAAG C

21

(2) INFORMATION FOR SEQ ID NO: 32:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 1140 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: CDS  
(B) LOCATION: 37..1089

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

GAGCAGTTAC GGTCTGTGTC CAGTGTAGAT GAAGTC	ATG ACT GTA CTC TAC CCA	54
	Met Thr Val Leu Tyr Pro	
	1 5	
GAA TAT TGG AAA ATG TAC AAG TGT CAG CTA AGG AAA GGA GGC TGG CAA		102
Glu Tyr Trp Lys Met Tyr Lys Cys Gln Leu Arg Lys Gly Gly Trp Gln		
10 15 20		
CAT AAC AGA GAA CAG GCC AAC CTC AAC TCA AGG ACA GAA GAG ACT ATA		150
His Asn Arg Glu Gln Ala Asn Leu Asn Ser Arg Thr Glu Glu Thr Ile		
25 30 35		
AAA TTT GCT GCA GCA CAT TAT AAT ACA GAG ATC TTG AAA AGT ATT GAT		198
Lys Phe Ala Ala Ala His Tyr Asn Thr Glu Ile Leu Lys Ser Ile Asp		
40 45 50		
AAT GAG TGG AGA AAG ACT CAA TGC ATG CCA CGG GAG GTG TGT ATA GAT		246
Asn Glu Trp Arg Lys Thr Gln Cys Met Pro Arg Glu Val Cys Ile Asp		
55 60 65 70		
GTG GGG AAG GAG TTT GGA GTC GCG ACA AAC ACC TTC TTT AAA CCT CCA		294
Val Gly Lys Glu Phe Gly Val Ala Thr Asn Thr Phe Phe Lys Pro Pro		
75 80 85		
TGT GTG TCC GTC TAC AGA TGT GGG GGT TGC TGC AAT AGT GAG GGG CTG		342
Cys Val Ser Val Tyr Arg Cys Gly Gly Cys Cys Asn Ser Glu Gly Leu		
90 95 100		
CAG TGC ATG AAC ACC AGC ACG AGC TAC CTC AGC AAG ACG TTA TTT GAA		390
Gln Cys Met Asn Thr Ser Thr Ser Tyr Leu Ser Lys Thr Leu Phe Glu		
105 110 115		
ATT ACA GTG CCT CTC TCT CAA GGC CCC AAA CCA GTA ACA ATC AGT TTT		438
Ile Thr Val Pro Leu Ser Gln Gly Pro Lys Pro Val Thr Ile Ser Phe		
120 125 130		
GCC AAT CAC ACT TCC TGC CGA TGC ATG TCT AAA CTG GAT GTT TAC AGA		486
Ala Asn His Thr Ser Cys Arg Cys Met Ser Lys Leu Asp Val Tyr Arg		
135 140 145 150		
CAA GTT CAT TCC ATT ATT AGA CGT TCC CTG CCA GCA ACA CTA CCA CAG		534
Gln Val His Ser Ile Ile Arg Arg Ser Leu Pro Ala Thr Leu Pro Gln		
155 160 165		
TGT CAG GCA GCG AAC AAG ACC TGC CCC ACC AAT TAC ATG TGG AAT AAT		582
Cys Gln Ala Ala Asn Lys Thr Cys Pro Thr Asn Tyr Met Trp Asn Asn		
170 175 180		
CAC ATC TGC AGA TGC CTG GCT CAG GAA GAT TTT ATG TTT TCC TCG GAT		630
His Ile Cys Arg Cys Leu Ala Gln Glu Asp Phe Met Phe Ser Ser Asp		
185 190 195		

-continued

GCT GGA GAT GAC TCA ACA GAT GGA TTC CAT GAC ATC TGT GGA CCA AAC	678
Ala Gly Asp Asp Ser Thr Asp Gly Phe His Asp Ile Cys Gly Pro Asn	
200 205 210	
AAG GAG CTG GAT GAA GAG ACC TGT CAG TGT GTC TGC AGA GCG GGG CTT	726
Lys Glu Leu Asp Glu Glu Thr Cys Gln Cys Val Cys Arg Ala Gly Leu	
215 220 225 230	
CGG CCT GCC AGC TGT GGA CCC CAC AAA GAA CTA GAC AGA AAC TCA TGC	774
Arg Pro Ala Ser Cys Gly Pro His Lys Glu Leu Asp Arg Asn Ser Cys	
235 240 245	
CAG TGT GTC TGT AAA AAC AAA CTC TTC CCC AGC CAA TGT GGG GCC AAC	822
Gln Cys Val Cys Lys Asn Lys Leu Phe Pro Ser Gln Cys Gly Ala Asn	
250 255 260	
CGA GAA TTT GAT GAA AAC ACA TGC CAG TGT GTA TGT AAA AGA ACC TGC	870
Arg Glu Phe Asp Glu Asn Thr Cys Gln Cys Val Cys Lys Arg Thr Cys	
265 270 275	
CCC AGA AAT CAA CCC CTA AAT CCT GGA AAA TGT GCC TGT GAA TGT ACA	918
Pro Arg Asn Gln Pro Leu Asn Pro Gly Lys Cys Ala Cys Glu Cys Thr	
280 285 290	
GAA AGT CCA CAG AAA TGC TTG TTA AAA GGA AAG TTC CAC CAC CAA	966
Glu Ser Pro Gln Lys Cys Leu Leu Lys Gly Lys Lys Phe His His Gln	
295 300 305 310	
ACA TGC AGC TGT TAC AGA CGG CCA TGT ACG AAC CGC CAG AAG GCT TGT	1014
Thr Cys Ser Cys Tyr Arg Arg Pro Cys Thr Asn Arg Gln Lys Ala Cys	
315 320 325	
GAG CCA GGA TTT TCA TAT AGT GAA GAA GTG TGT CGT TGT GTC CCT TCA	1062
Glu Pro Gly Phe Ser Tyr Ser Glu Glu Val Cys Arg Cys Val Pro Ser	
330 335 340	
TAT TGG AAA AGA CCA CAA ATG AGC TAAGATTGTA CTGTTTTCCTA GTTCATCGA	1116
Tyr Trp Lys Arg Pro Gln Met Ser	
345 350	
TTTCTATTAT GGAAAACTGT GTTG	1140

## (2) INFORMATION FOR SEQ ID NO: 33:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 350 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

Met Thr Val Leu Tyr Pro Glu Tyr Trp Lys Met Tyr Lys Cys Gln Leu	1 5 10 15
Arg Lys Gly Gly Trp Gln His Asn Arg Glu Gln Ala Asn Leu Asn Ser	20 25 30
Arg Thr Glu Glu Thr Ile Lys Phe Ala Ala Ala His Tyr Asn Thr Glu	35 40 45
Ile Leu Lys Ser Ile Asp Asn Glu Trp Arg Lys Thr Gln Cys Met Pro	50 55 60
Arg Glu Val Cys Ile Asp Val Gly Lys Glu Phe Gly Val Ala Thr Asn	65 70 75 80
Thr Phe Phe Lys Pro Pro Cys Val Ser Val Tyr Arg Cys Gly Gly Cys	85 90 95
Cys Asn Ser Glu Gly Leu Gln Cys Met Asn Thr Ser Thr Ser Tyr Leu	100 105 110
Ser Lys Thr Leu Phe Glu Ile Thr Val Pro Leu Ser Gln Gly Pro Lys	115 120 125
Pro Val Thr Ile Ser Phe Ala Asn His Thr Ser Cys Arg Cys Met Ser	

-continued

130	135	140
Lys Leu Asp Val Tyr Arg Gln Val His Ser Ile Ile Arg Arg Ser Leu		
145	150	155 160
Pro Ala Thr Leu Pro Gln Cys Gln Ala Ala Asn Lys Thr Cys Pro Thr		
	165	170 175
Asn Tyr Met Trp Asn Asn His Ile Cys Arg Cys Leu Ala Gln Glu Asp		
	180	185 190
Phe Met Phe Ser Ser Asp Ala Gly Asp Asp Ser Thr Asp Gly Phe His		
	195	200 205
Asp Ile Cys Gly Pro Asn Lys Glu Leu Asp Glu Glu Thr Cys Gln Cys		
	210	215 220
Val Cys Arg Ala Gly Leu Arg Pro Ala Ser Cys Gly Pro His Lys Glu		
	225	230 235 240
Leu Asp Arg Asn Ser Cys Gln Cys Val Cys Lys Asn Lys Leu Phe Pro		
	245	250 255
Ser Gln Cys Gly Ala Asn Arg Glu Phe Asp Glu Asn Thr Cys Gln Cys		
	260	265 270
Val Cys Lys Arg Thr Cys Pro Arg Asn Gln Pro Leu Asn Pro Gly Lys		
	275	280 285
Cys Ala Cys Glu Cys Thr Glu Ser Pro Gln Lys Cys Leu Leu Lys Gly		
	290	295 300
Lys Lys Phe His His Gln Thr Cys Ser Cys Tyr Arg Arg Pro Cys Thr		
	305	310 315 320
Asn Arg Gln Lys Ala Cys Glu Pro Gly Phe Ser Tyr Ser Glu Glu Val		
	325	330 335
Cys Arg Cys Val Pro Ser Tyr Trp Lys Arg Pro Gln Met Ser		
	340	345 350

## (2) INFORMATION FOR SEQ ID NO: 34:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1997 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

- (ix) FEATURE:  
 (A) NAME/KEY: CDS  
 (B) LOCATION: 352..1608

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

CCCCCCCCCG CTCTCCAAAA AGCTACACCG ACGCGGACCG CGCGGGCGTC CTCCTCGCC	60
CTCGCTTCAC CTCGGGGGCT CCGAATGCGG GGAGCTCGGA TGTCCGGTTT CCTGTGAGG	120
TTTACCTGA CACCGGCGCG CTTCGCCCGG CACTGGCTGG GAGGGCGCCC TGCAAAGTT	180
GGAACGCGGA GCGCGGACG CGCTCCCGCC GCCTCCGGCT CGCCAGGGG GGGTGGCCG	240
GAGGAGCCCG GGGGAGAGGG ACCAGGAGGG GCCCGCGGCC TCGCAGGGGC GCGCGCGCC	300
CCACCCCTGC CCGCGCCAGC GGACCGGTCC CCCACCCCGG GTCCTTCAC C ATG CAC	357
	Met His
	1
TTG CTG GGC TTC TTC TCT GTG GCG TGT TCT CTG CTC GCC GCT GCG CTG	405
Leu Leu Gly Phe Phe Ser Val Ala Cys Ser Leu Leu Ala Ala Ala Leu	
	5 10 15
CTC CCG GGT CCT CGC GAG GCG CCC GCC GCC GCC GCC TTC GAG TCC	453
Leu Pro Gly Pro Arg Glu Ala Pro Ala Ala Ala Ala Phe Glu Ser	
	20 25 30

-continued

GGA CTC GAC CTC TCG GAC GCG GAG CCC GAC GCG GGC GAG GCC ACG GCT Gly Leu Asp Leu Ser Asp Ala Glu Pro Asp Ala Gly Glu Ala Thr Ala 35 40 45 50	501
TAT GCA AGC AAA GAT CTG GAG GAG CAG TTA CGG TCT GTG TCC AGT GTA Tyr Ala Ser Lys Asp Leu Glu Glu Gln Leu Arg Ser Val Ser Ser Val 55 60 65	549
GAT GAA CTC ATG ACT GTA CTC TAC CCA GAA TAT TGG AAA ATG TAC AAG Asp Glu Leu Met Thr Val Leu Tyr Pro Glu Tyr Trp Lys Met Tyr Lys 70 75 80	597
TGT CAG CTA AGG AAA GGA GGC TGG CAA CAT AAC AGA GAA CAG GCC AAC Cys Gln Leu Arg Lys Gly Gly Trp Gln His Asn Arg Glu Gln Ala Asn 85 90 95	645
CTC AAC TCA AGG ACA GAA GAG ACT ATA AAA TTT GCT GCA GCA CAT TAT Leu Asn Ser Arg Thr Glu Thr Ile Lys Phe Ala Ala Ala His Tyr 100 105 110	693
AAT ACA GAG ATC TTG AAA AGT ATT GAT AAT GAG TGG AGA AAG ACT CAA Asn Thr Glu Ile Leu Lys Ser Ile Asp Asn Glu Trp Arg Lys Thr Gln 115 120 125 130	741
TGC ATG CCA CGG GAG GTG TGT ATA GAT GTG GGG AAG GAG TTT CGA GTC Cys Met Pro Arg Glu Val Cys Ile Asp Val Gly Lys Glu Phe Gly Val 135 140 145	789
GCG ACA AAC ACC TTC TTT AAA CCT CCA TGT GTG TCC GTC TAC AGA TGT Ala Thr Asn Thr Phe Phe Lys Pro Pro Cys Val Ser Val Tyr Arg Cys 150 155 160	837
GGG GGT TGC TGC AAT AGT GAG GGG CTG CAG TGC ATG AAC ACC AGC ACG Gly Gly Cys Cys Asn Ser Glu Gly Leu Gln Cys Met Asn Thr Ser Thr 165 170 175	885
AGC TAC CTC AGC AAG ACG TTA TTT GAA ATT ACA GTG CCT CTC TCT CAA Ser Tyr Leu Ser Lys Thr Leu Phe Glu Ile Thr Val Pro Leu Ser Gln 180 185 190	933
GGC CCC AAA CCA GTA ACA ATC AGT TTT GCC AAT CAC ACT TCC TGC CGA Gly Pro Lys Pro Val Thr Ile Ser Phe Ala Asn His Thr Ser Cys Arg 195 200 205 210	981
TGC ATG TCT AAA CTG GAT GTT TAC AGA CAA GTT CAT TCC ATT ATT AGA Cys Met Ser Lys Leu Asp Val Tyr Arg Gln Val His Ser Ile Ile Arg 215 220 225	1029
CGT TCC CTG CCA GCA ACA CTA CCA CAG TGT CAG GCA GCG AAC AAG ACC Arg Ser Leu Pro Ala Thr Leu Pro Gln Cys Gln Ala Ala Asn Lys Thr 230 235 240	1077
TGC CCC ACC AAT TAC ATG TGG AAT AAT CAC ATC TGC AGA TGC CTG GCT Cys Pro Thr Asn Tyr Met Trp Asn Asn His Ile Cys Arg Cys Leu Ala 245 250 255	1125
CAG GAA GAT TTT ATG TTT TCC TCG GAT GCT GGA GAT GAC TCA ACA GAT Gln Glu Asp Phe Met Phe Ser Ser Asp Ala Gly Asp Asp Ser Thr Asp 260 265 270	1173
GGA TTC CAT GAC ATC TGT GGA CCA AAC AAG GAG CTG GAT GAA GAG ACC Gly Phe His Asp Ile Cys Gly Pro Asn Lys Glu Leu Asp Glu Glu Thr 275 280 285 290	1221
TGT CAG TGT GTC TGC AGA GCG GGG CTT CGG CCT GCC AGC TGT GGA CCC Cys Gln Cys Val Cys Arg Ala Gly Leu Arg Pro Ala Ser Cys Gly Pro 295 300 305	1269
CAC AAA GAA CTA GAC AGA AAC TCA TGC CAG TGT GTC TGT AAA AAC AAA His Lys Glu Leu Asp Arg Asn Ser Cys Gln Cys Val Cys Lys Asn Lys 310 315 320	1317
CTC TTC CCC AGC CAA TGT GGG GCC AAC CGA GAA TTT GAT GAA AAC ACA Leu Phe Pro Ser Gln Cys Gly Ala Asn Arg Glu Phe Asp Glu Asn Thr 325 330 335	1365
TGC CAG TGT GTA TGT AAA AGA ACC TGC CCC AGA AAT CAA CCC CTA AAT Cys Gln Cys Val Cys Lys Arg Thr Cys Pro Arg Asn Gln Pro Leu Asn 340 345 350	1413

-continued

340	345	350	
GCT GGA AAA TGT GCC TGT GAA TGT ACA GAA AGT CCA CAG AAA TGC TTG			1461
Pro Gly Lys Cys Ala Cys Glu Cys Thr Glu Ser Pro Gln Lys Cys Leu			
355	360	365	370
TTA AAA GGA AAG AAG TTC CAC CAC CAA ACA TGC AGC TGT TAC AGA CGG			1509
Leu Lys Gly Lys Lys Phe His His Gln Thr Cys Ser Cys Tyr Arg Arg			
375	380		385
CCA TGT ACG AAC CGC CAG AAG GCT TGT GAG CCA GGA TTT TCA TAT AGT			1557
Pro Cys Thr Asn Arg Gln Lys Ala Cys Glu Pro Gly Phe Ser Tyr Ser			
390	395		400
GAA GAA GTG TGT CGT TGT GTC CCT TCA TAT TGG AAA AGA CCA CAA ATG			1605
Glu Glu Val Cys Arg Cys Val Pro Ser Tyr Trp Lys Arg Pro Gln Met			
405	410		415
AGC TAAGATTGTA CTGTTTTCCA GTTCATCGAT TTTCTATTAT GGAAAACTGT			1658
Ser			
GTTCGCACAG TAGAACTGTC TGTGAACAGA GAGACCCCTG TGGGTCCATG CTAACAAA			1718
CAAAAGTCTG TCTTTCCTGA ACCATGTGGA TAACTTTACA GAAATGGACT GGAGCTCA			1778
TGCAAAAGGC CTCTTGTAAG GACTGGTTTT CTGCCAATGA CCAAAACAGCC AAGATTTT			1838
TCTGTGATT TCTTTAAAG AATGACTATA TAATTTATTT CCACTAAAAA TATTGTTT			1898
GCATTCATTT TTATAGCAAC AACAATGGT AAAACTCACT GTGATCAATA TTTTATA			1958
ATGCAAAATA TGTTTAAAT AAAATGAAAA TTGTATTAT			1997

## (2) INFORMATION FOR SEQ ID NO: 35:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 419 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

Met His Leu Leu Gly Phe Phe Ser Val Ala Cys Ser Leu Leu Ala Ala  
 1 5 10 15

Ala Leu Leu Pro Gly Pro Arg Glu Ala Pro Ala Ala Ala Ala Phe  
 20 25 30

Glu Ser Gly Leu Asp Leu Ser Asp Ala Glu Pro Asp Ala Gly Glu Ala  
 35 40 45

Thr Ala Tyr Ala Ser Lys Asp Leu Glu Glu Gln Leu Arg Ser Val Ser  
 50 55 60

Ser Val Asp Glu Leu Met Thr Val Leu Tyr Pro Glu Tyr Trp Lys Met  
 65 70 75 80

Tyr Lys Cys Gln Leu Arg Lys Gly Gly Trp Gln His Asn Arg Glu Gln  
 85 90 95

Ala Asn Leu Asn Ser Arg Thr Glu Glu Thr Ile Lys Phe Ala Ala Ala  
 100 105 110

His Tyr Asn Thr Glu Ile Leu Lys Ser Ile Asp Asn Glu Trp Arg Lys  
 115 120 125

Thr Gln Cys Met Pro Arg Glu Val Cys Ile Asp Val Gly Lys Glu Phe  
 130 135 140

Gly Val Ala Thr Asn Thr Phe Phe Lys Pro Pro Cys Val Ser Val Tyr  
 145 150 155 160

Arg Cys Gly Gly Cys Cys Asn Ser Glu Gly Leu Gln Cys Met Asn Thr  
 165 170 175

Ser Thr Ser Tyr Leu Ser Lys Thr Leu Phe Glu Ile Thr Val Pro Leu

-continued

180						185						190					
Ser	Gln	Gly	Pro	Lys	Pro	Val	Thr	Ile	Ser	Phe	Ala	Asn	His	Thr	Ser		
		195					200					205					
Cys	Arg	Cys	Met	Ser	Lys	Leu	Asp	Val	Tyr	Arg	Gln	Val	His	Ser	Ile		
	210					215					220						
Ile	Arg	Arg	Ser	Leu	Pro	Ala	Thr	Leu	Pro	Gln	Cys	Gln	Ala	Ala	Asn		
	225				230					235					240		
Lys	Thr	Cys	Pro	Thr	Asn	Tyr	Met	Trp	Asn	Asn	His	Ile	Cys	Arg	Cys		
				245					250					255			
Leu	Ala	Gln	Glu	Asp	Phe	Met	Phe	Ser	Ser	Asp	Ala	Gly	Asp	Asp	Ser		
			260					265					270				
Thr	Asp	Gly	Phe	His	Asp	Ile	Cys	Gly	Pro	Asn	Lys	Glu	Leu	Asp	Glu		
		275					280					285					
Glu	Thr	Cys	Gln	Cys	Val	Cys	Arg	Ala	Gly	Leu	Arg	Pro	Ala	Ser	Cys		
	290					295					300						
Gly	Pro	His	Lys	Glu	Leu	Asp	Arg	Asn	Ser	Cys	Gln	Cys	Val	Cys	Lys		
	305				310					315					320		
Asn	Lys	Leu	Phe	Pro	Ser	Gln	Cys	Gly	Ala	Asn	Arg	Glu	Phe	Asp	Glu		
			325						330					335			
Asn	Thr	Cys	Gln	Cys	Val	Cys	Lys	Arg	Thr	Cys	Pro	Arg	Asn	Gln	Pro		
			340					345					350				
Leu	Asn	Pro	Gly	Lys	Cys	Ala	Cys	Glu	Cys	Thr	Glu	Ser	Pro	Gln	Lys		
		355					360					365					
Cys	Leu	Leu	Lys	Gly	Lys	Lys	Phe	His	His	Gln	Thr	Cys	Ser	Cys	Tyr		
	370					375					380						
Arg	Arg	Pro	Cys	Thr	Asn	Arg	Gln	Lys	Ala	Cys	Glu	Pro	Gly	Phe	Ser		
	385				390					395					400		
Tyr	Ser	Glu	Glu	Val	Cys	Arg	Cys	Val	Pro	Ser	Tyr	Trp	Lys	Arg	Pro		
				405					410					415			
Gln	Met	Ser															

What is claimed is:

1. A purified and isolated polypeptide capable of binding to an Flt4 receptor tyrosine kinase, said polypeptide comprising a portion of amino acids 1-180 of SEQ ID NO: 33 effective to permit such binding, said polypeptide lacking all of amino acids of SEQ ID NO: 33 beyond position 180.

2. A pharmaceutical composition comprising a polypeptide according to claim 1 in a pharmaceutically-acceptable diluent, adjuvant, or carrier.

3. A method of modulating the activity of human Flt4 receptor tyrosine kinase comprising administering to a person in need of modulation of Flt4 receptor tyrosine kinase activity a composition according to claim 2.

4. A polypeptide according to claim 1 further comprising a detectable label.

5. A purified and isolated polypeptide according to claim 1 that binds Flt4 and stimulates Flt4 phosphorylation in mammalian cells expressing Flt4.

6. A method of modulating the activity of human Flt4 receptor tyrosine kinase comprising contacting cells that express human Flt4 receptor tyrosine kinase with a polypeptide according to claim 1.

7. A purified and isolated polypeptide capable of binding with high affinity to the extracellular domain of Flt4 receptor tyrosine kinase (Flt4), wherein the polypeptide comprises a portion of SEQ ID NO: 33 effective to permit such binding,

and wherein the polypeptide has an apparent molecular weight of approximately 23 kD as assessed by SDS-PAGE under reducing conditions.

8. A pharmaceutical composition comprising a polypeptide according to claim 7 in a pharmaceutically-acceptable diluent, adjuvant, or carrier.

9. A method of modulating the activity of human Flt4 receptor tyrosine kinase comprising administering to a person in need of modulation of Flt4 receptor tyrosine kinase activity a composition according to claim 8.

10. A purified and isolated polypeptide according to claim 7 that binds Flt4 and stimulates Flt4 phosphorylation in mammalian cells expressing Flt4.

11. A method of modulating the activity of human Flt4 receptor tyrosine kinase comprising contacting cells that express human Flt4 receptor tyrosine kinase with a polypeptide according to claim 10.

12. A purified and isolated polypeptide according to claim 10, said polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 13.

13. A polypeptide according to claim 7 further comprising a detectable label.

14. A polypeptide according to claim 7 wherein said portion of SEQ ID NO: 33 effective to permit such binding is a continuous portion of SEQ ID NO: 33 within amino acids 1-180 of SEQ ID NO: 33.



15. A polypeptide according to claim 7, wherein the amino terminus of said portion effective to permit such binding corresponds with position 34 of SEQ ID NO: 33.

16. A method of modulating the activity of human Flt4 receptor tyrosine kinase comprising contacting cells that express human Flt4 receptor tyrosine kinase with a polypeptide according to claim 7.

17. A purified and isolated polypeptide comprising a human polypeptide capable of binding with high affinity to the extracellular domain of Flt4 receptor tyrosine kinase and having an apparent molecular weight of approximately 23 kD as assessed by SDS-PAGE under reducing conditions, wherein amino terminal amino acids 2 through 18 of said human polypeptide have an amino acid sequence corresponding to amino acids 2 through 18 set forth in SEQ ID NO: 13.

18. A method of modulating the activity of human Flt4 receptor tyrosine kinase comprising contacting cells that express human Flt4 receptor tyrosine kinase with a polypeptide according to claim 17.

19. A purified and isolated polypeptide according to claim 17 that binds Flt4 and stimulates Flt4 phosphorylation in mammalian cells expressing Flt4.

20. A pharmaceutical composition comprising a polypeptide according to claim 17 in a pharmaceutically-acceptable diluent, adjuvant, or carrier.

21. A method of modulating the activity of human Flt4 receptor tyrosine kinase comprising administering to a person in need of modulation of Flt4 receptor tyrosine kinase activity a composition according to claim 20.

22. A polypeptide according to claim 17 further comprising a detectable label.

23. A purified and isolated polypeptide capable of binding with high affinity to the extracellular domain of Flt4 receptor tyrosine kinase, wherein said polypeptide has an apparent molecular weight of approximately 23 kD as assessed by SDS-PAGE under reducing conditions and is purifiable from conditioned media from a PC-3 prostatic adenocarcinoma cell line, said cell line having ATCC CRL No. 1435, using an affinity purification procedure wherein the affinity purification matrix comprises a polypeptide comprising the extracellular domain of Flt4 receptor tyrosine kinase.

24. A polypeptide according to claim 23 which is capable of stimulating Flt4 phosphorylation in mammalian cells expressing Flt4 receptor tyrosine kinase.

25. A method of modulating the activity of human Flt4 receptor tyrosine kinase comprising contacting cells that express human Flt4 receptor tyrosine kinase with a polypeptide according to claim 24.

26. A polypeptide according to claim 23 further comprising a detectable label.

27. A pharmaceutical composition comprising a polypeptide according to claim 23 in a pharmaceutically-acceptable diluent, adjuvant, or carrier.

28. A method of modulating the activity of human Flt4 receptor tyrosine kinase comprising administering to a person in need of modulation of Flt4 receptor tyrosine kinase activity a composition according to claim 27.

29. A method of modulating the activity of human Flt4 receptor tyrosine kinase comprising contacting cells that express human Flt4 receptor tyrosine kinase with a polypeptide according to claim 23.

\* \* \* \* \*

AUSTRALIA

*Patents Act 1990*

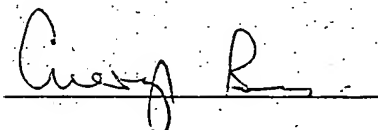
IN THE MATTER OF Australian Patent  
Application Serial No: 696764 by Human  
Genome Sciences, Inc.

-and-

IN THE MATTER OF Opposition thereto by  
Ludwig Institute for Cancer Research

THIS IS Exhibit PAWR-14  
referred to in the Statutory Declaration  
of Peter Adrian Walton Rogers  
made before me

DATED this 12<sup>th</sup> Day of November, 2001



(Signature of Witness)

Medical Practitioner



US006245530B1

(12) **United States Patent**  
Alitalo et al.

(10) Patent No.: **US 6,245,530 B1**  
(45) Date of Patent: **Jun. 12, 2001**

(54) **RECEPTOR LIGAND**

(75) Inventors: Kari Alitalo, Espoo (FI); Vladimir Joukov, Boston, MA (US)

(73) Assignees: Ludwig Institute for Cancer Research, New York, NY (US); Helsinki University Licensing, Ltd. OY, Helsinki (FI)

(\*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: 08/585,895

(22) Filed: Jan. 12, 1996

**Related U.S. Application Data**

(63) Continuation-in-part of application No. 08/510,133, filed on Aug. 1, 1995.

(51) Int. Cl.<sup>7</sup> ..... C12N 15/12; C12N 15/63; C12N 5/10; C12N 5/16

(52) U.S. Cl. .... 435/69.4; 435/70.1; 435/325; 435/320.1; 536/23.51; 530/399; 935/13

(58) Field of Search ..... 536/23.51; 435/252.3; 435/254.11; 320.1; 419; 69.4; 70.1; 325; 530/399; 935/13

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A2	4/1996	(WO)
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(List continued on next page.)

Primary Examiner—Christine Saoud  
 (74) Attorney, Agent, or Firm—Marshall, O'Toole, Gerstein, Murray & Borun

(57) **ABSTRACT**

Provided are ligands for the receptor tyrosine kinase, Flt4. Also provided are cDNAs and vectors encoding the ligands, pharmaceutical compositions and diagnostic reagents.

35 Claims, 30 Drawing Sheets

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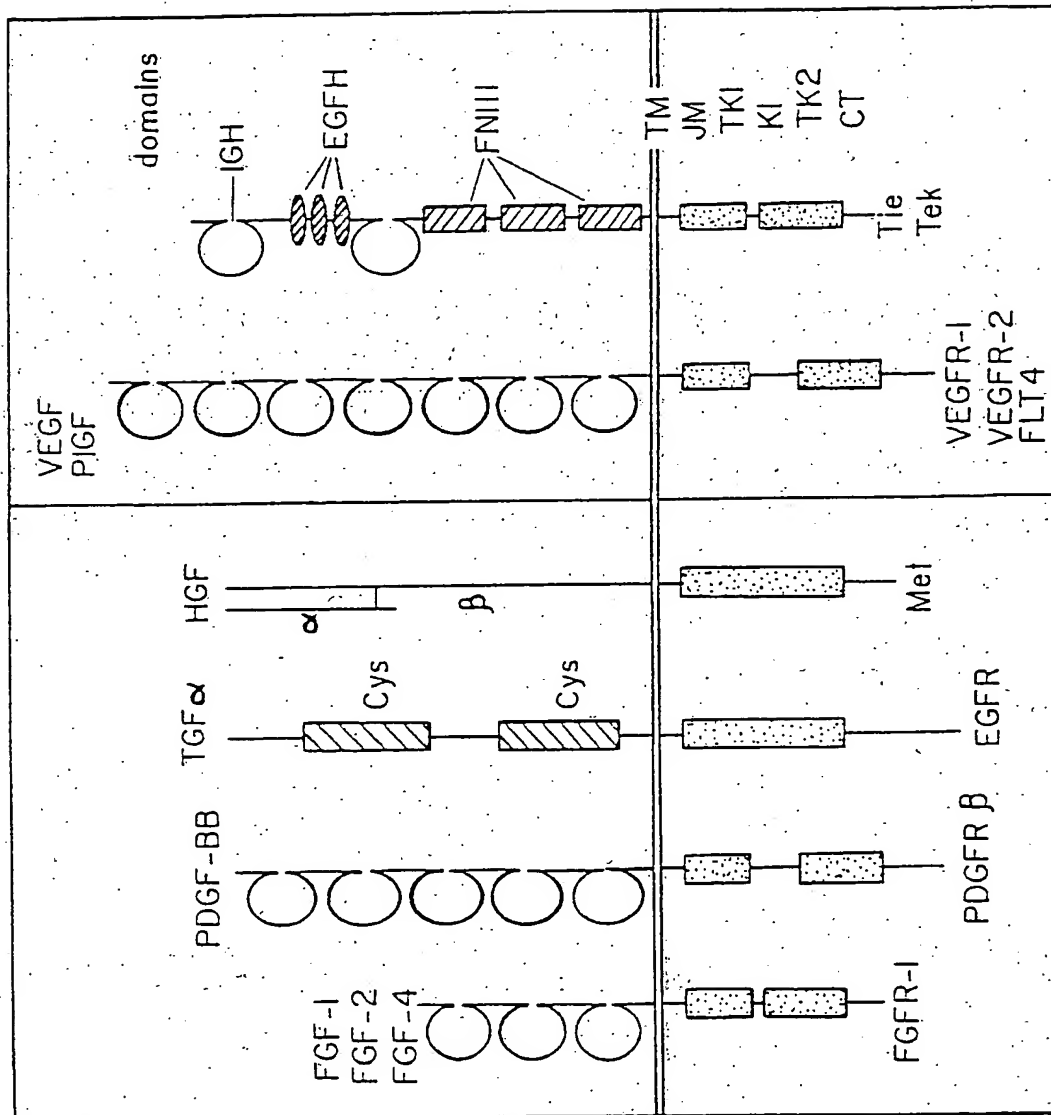


FIGURE 1



FIGURE 2A

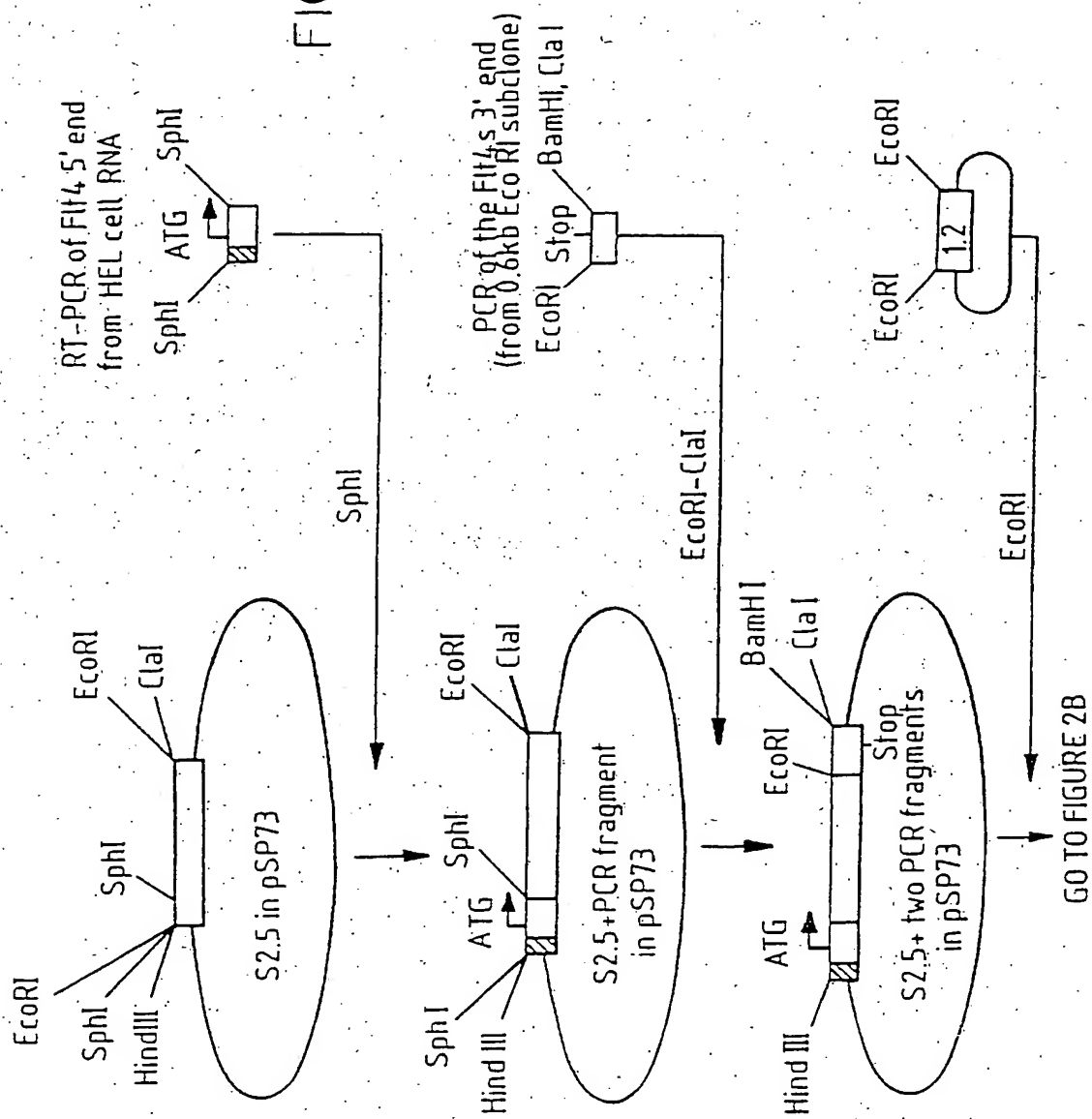
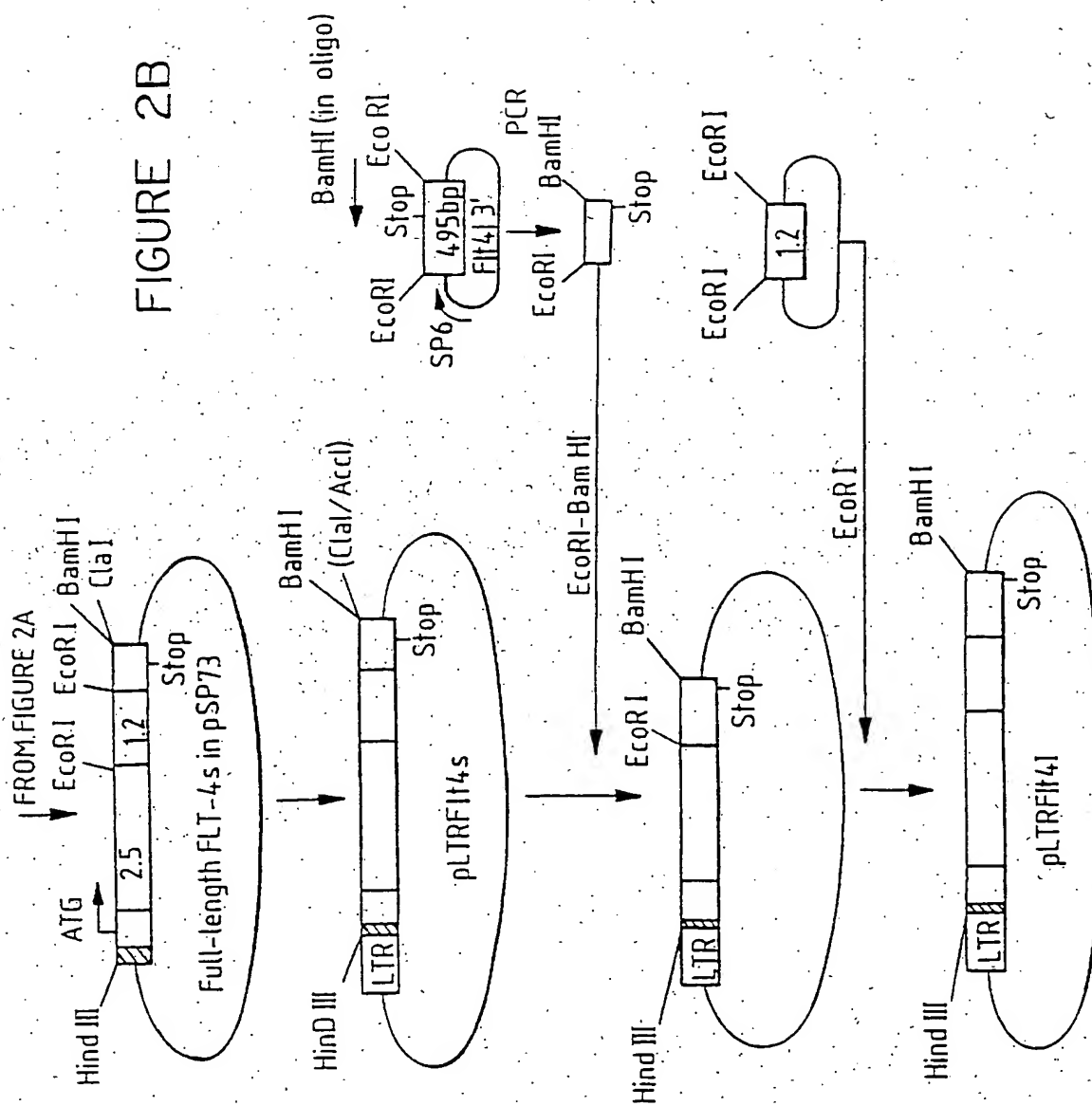


FIGURE 2B



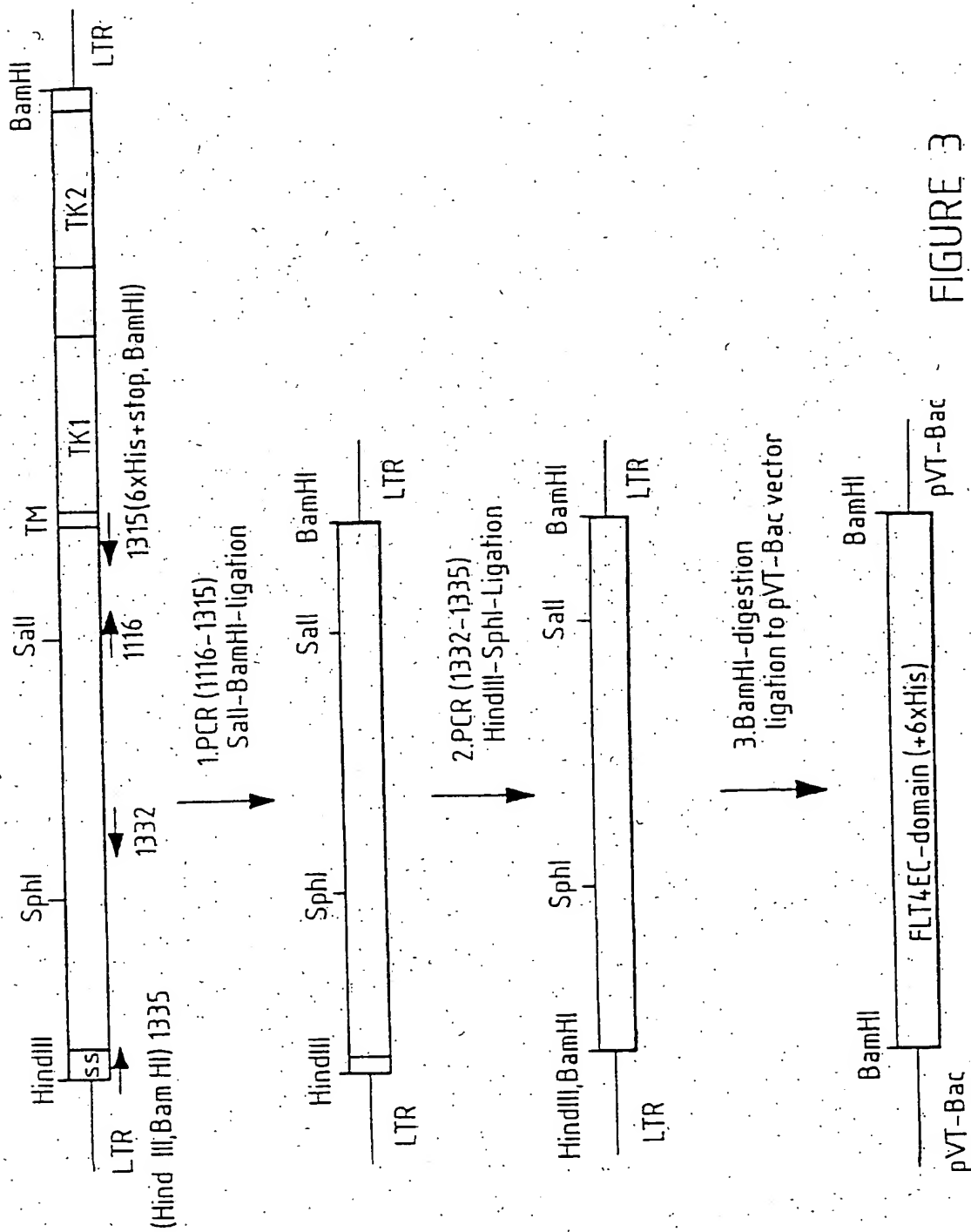


FIGURE 3

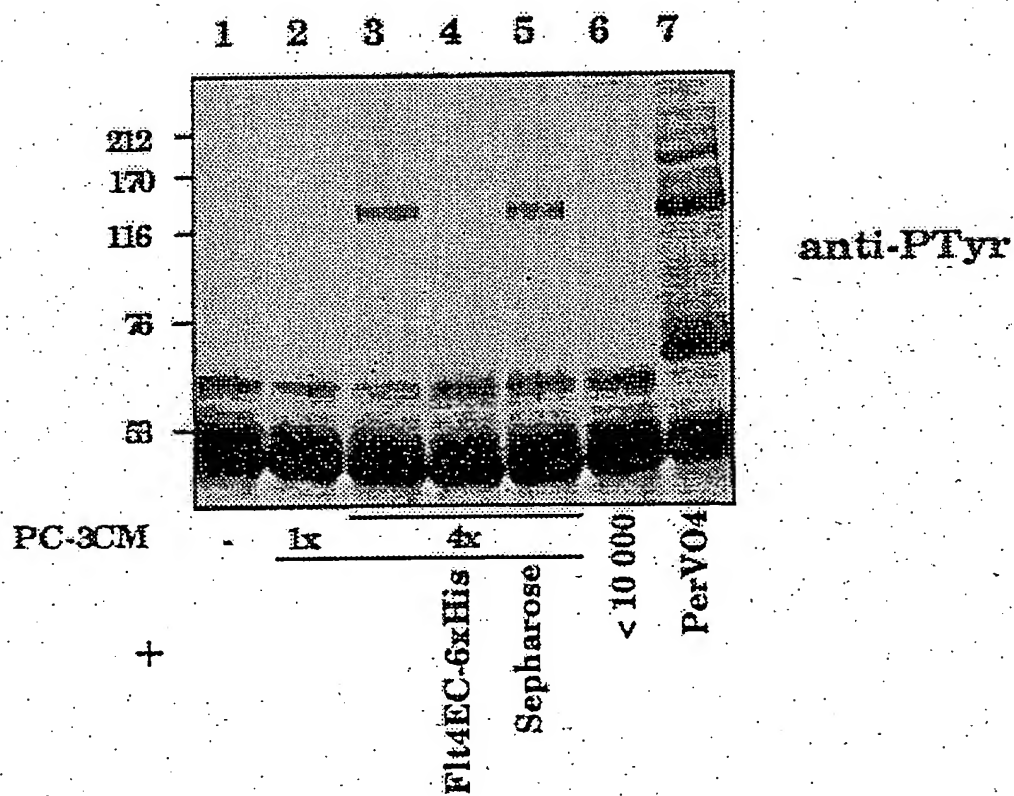


FIG. 4

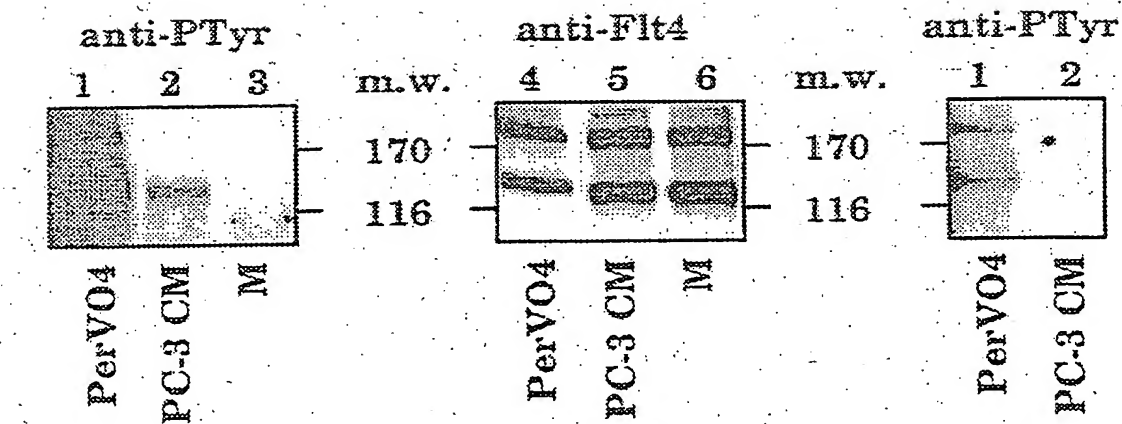


FIG. 5A

FIG. 5B

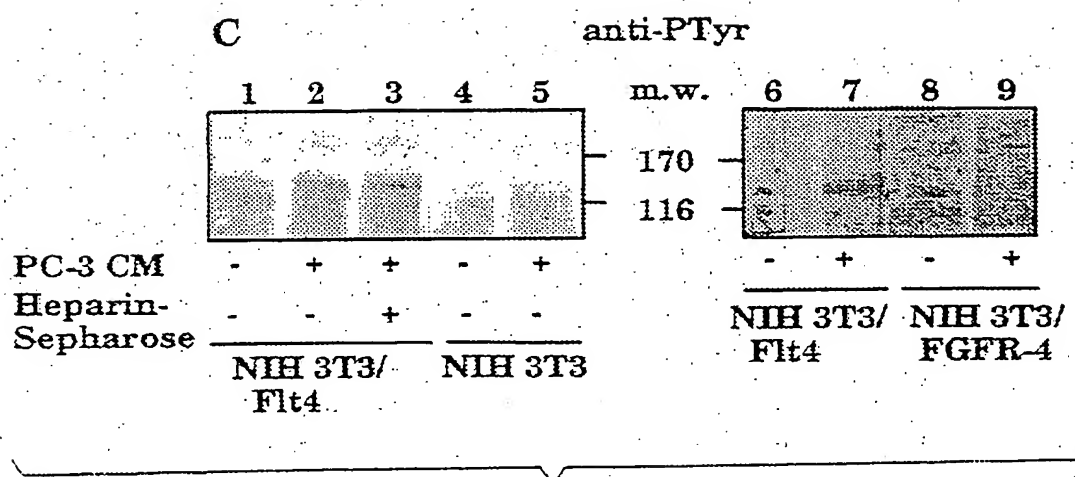


FIG. 5C

FIG. 6

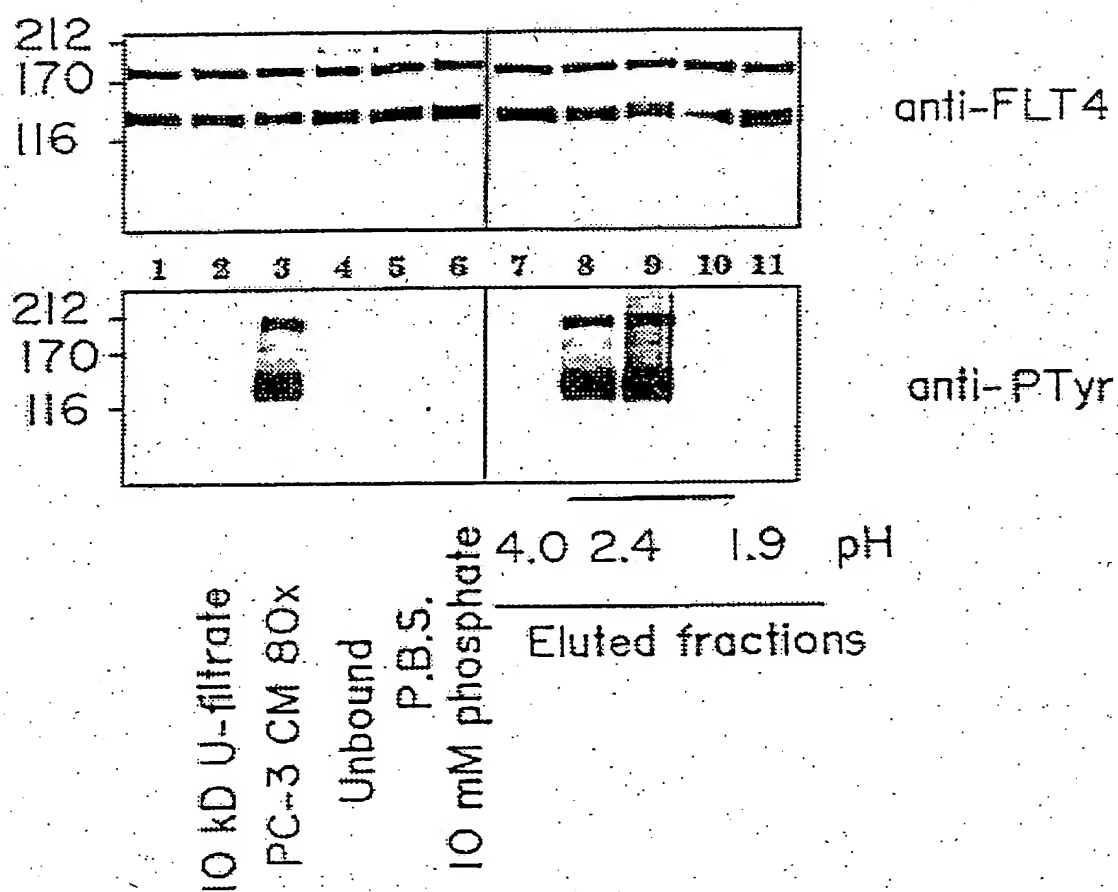
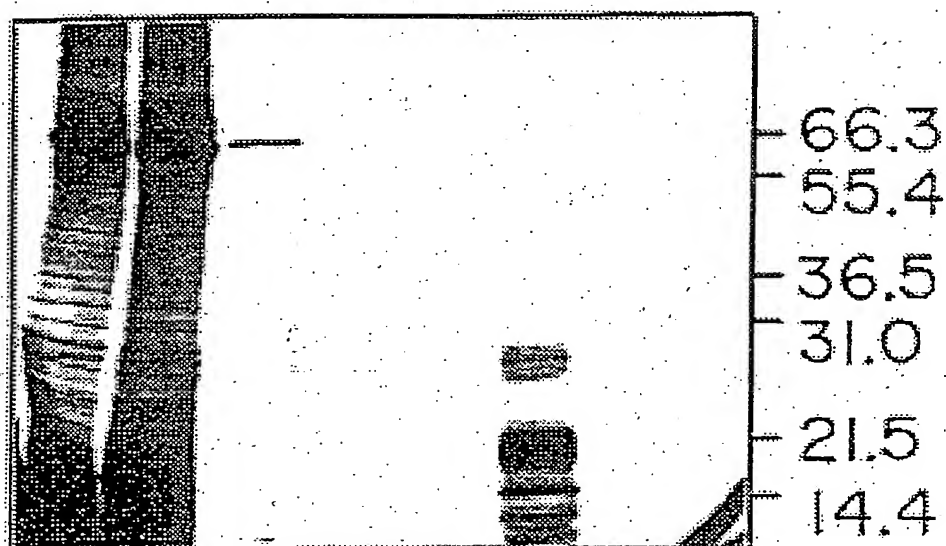


FIG. 7



PC-3 CM 80x

Unbound

P.B.S.

10 mM phosphate

4.0 2.4 1.9

Eluted  
fractions

M.w. standards



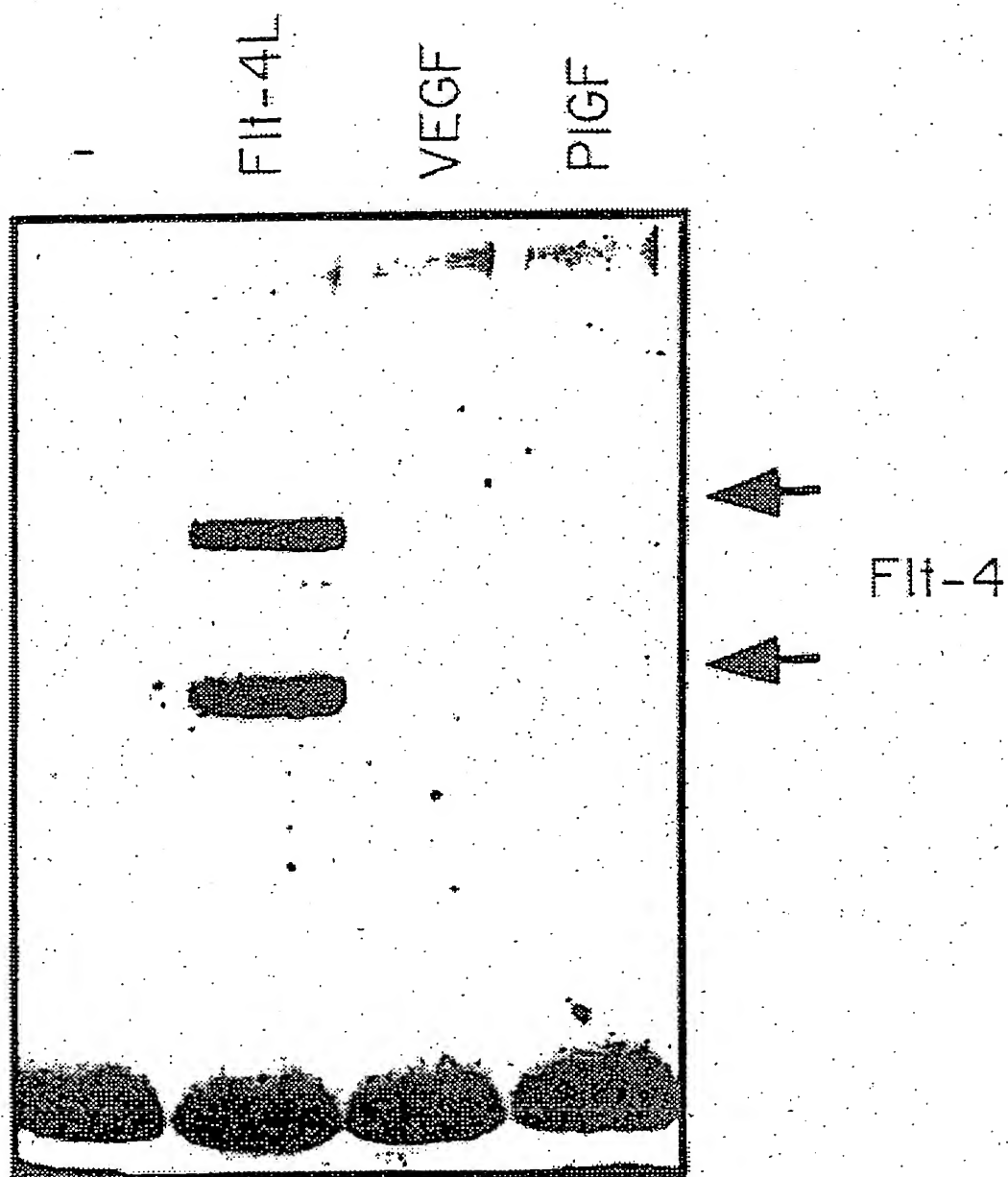


FIG. 8

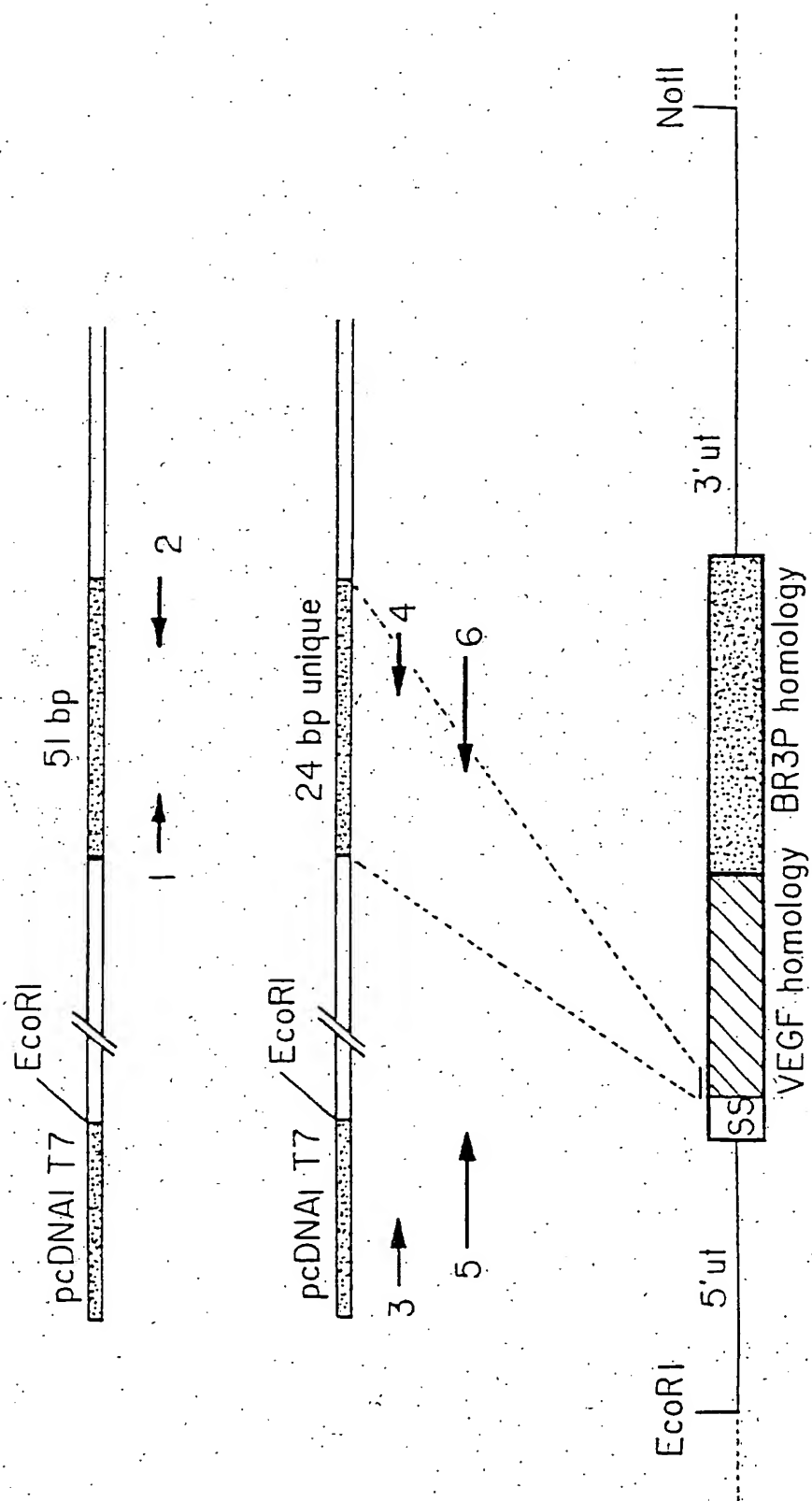


FIG. 9A

MetThrValLeuTyrProGluTyr  
GAGCAGTTACGGTCTGTGTCCAGTGTAGATGAACCTCATGACTGTACTCTACCCAGAATAT  
10 30 50

TrpLysMetTyrLysCysGlnLeuArgLysGlyGlyTrpGlnHisAsnArgGluGlnAla  
TGGAAAAATGTACAAAGTGTCAAGCTAAGGAAAGGAGGCTGGCAACATAACAGAGAACAGGCC  
70 90 110

AsnLeuAsnSerArgThrGluGluThrIleLysPheAlaAlaHisTyrAsnThrGlu  
AACCTCAACTCAAGGACAGAGAGACTATAAAATTTGCTGCAGCACATTATAATACAGAG  
130 150 170

IleLeuLysSerIleAspAsnGluTrpArgLysThrGlnCysMetProArgGluValCys  
ATCTTGAAAAGTATTGATAATGAGTGGAGAAAGACTCAATGCATGCCACGGGAGGTGTGT  
190 210 230

IleAspValGlyLysGluPheGlyValAlaThrAsnThrPhePheLysProProCysVal  
ATAGATGTGGGAAGGAGTTTGGAGTCGCGACAAACACCTTCTTTAAACCTCCATGTGTG  
250 270 290

SerValTyrArgCysGlyGlyCysCysAsnSerGluGlyLeuGlnCysMetAsnThrSer  
TCCGTCTACAGATGTGGGGGTTGCTGCAATAGTGAGGGGCTGCAGTGCATGAACACCAGC  
310 330 350

FIG. 9B

ThrSerTyrLeuSerLysThrLeuPheGluIleThrValProLeuSerGlnGlyProLys  
ACGAGCTACCTCAGCAAGACGTTATTTGAAATTACAGTGCCTCTCTCTCAAGGCCCAAA  
370 390 410

ProValThrIleSerPheAlaAsnHisThrSerCysArgCysMetSerLysLeuAspVal  
CCAGTAACAATCAGTTTGGCCAATCACACTTCCTGCCGATGCATGTCTAAACTGGATGTT  
430 450 470

TyrArgGlnValHisSerIleIleArgArgSerLeuProAlaThrLeuProGlnCysGln  
TACAGACAAGTTCATTCCATTATTAGACGTTCCCTGCCAGCAACACTACCACAGTGTCTCAG  
490 510 530

AlaAlaAsnLysThrCysProThrAsnTyrMetTrpAsnAsnHisIleCysArgCysLeu  
GCAGCGAACAAAGACCTGCCCCACCAATTACATGTGGAATAATCACATCTGCAGATGCCTG  
550 570 590

AlaGlnGluAspPheMetPheSerSerAspAlaGlyAspAspSerThrAspGlyPheHis  
GCTCAGGAAGATTTTATGTTTCCCTCGGATGCTGGAGATGACTCAACAGATGGATTCCAT  
610 630 650

AspIleCysGlyProAsnLysGluLeuAspGluGluThrCysGlnCysValCysArgAla  
GACATCTGTGGACCAACAAGGAGCTGGATGAAGAGACCCTGTCTAGTGTGTCTGCAGAGCG  
670 690 710

GlyLeuArgProAlaSerCysGlyProHisLysGluLeuAspArgAsnSerCysGlnCys  
GGGCTTCGGCCTGCCAGCTGTGGACCCCAAGAACTAGACAGAAACTCATGCCCAGTGT  
730 750 770

FIG. 9C

ValCysLysAsnLysLeuPheProSerGlnCysGlyAlaAsnArgGluPheAspGluAsn  
GTCTGTAAACAAACTCTTCCCCAGCCCAATGTGGGGCCCAACCGAGAAATTGTATGAAAC  
790 810 830

ThrCysGlnCysValCysLysArgThrCysProArgAsnGlnProLeuAsnProGlyLys  
ACATGCCAGTGTGTATGTAAAGAACCTGCCCCAGAAATCAACCCCTAAATCCTCGAAAAA  
850 870 890

CysAlaCysGluCysThrGluSerProGlnLysCysLeuLeuLysGlyLysLysPheHis  
TGTGCCTGTGAATGTACAGAAAGTCCACAGAAATGCTTGTAAAGGAAAGAGTTCCAC  
910 930 950

HisGlnThrCysSerCysTyrArgArgProCysThrAsnArgGlnLysAlaCysGluPro  
CACCAACATGCAGCTGTTACAGACGGCCATGTACGAACCGCCAGAGGCTTGTGAGCCA  
970 990 1010

GlyPheSerTyrSerGluGluValCysArgCysValProSerTyrTrpLysArgProGln  
GGATTTTCATATAGTGAAGAAGTGTGTCGTTGTGTCCCTTCATATTTGAAAAAGACCACAA  
1030 1050 1070

MetSerEnd  
ATGAGCTAAGATTGTACTGTTTCCAGTTCATCGATTTTCTATTATGAAAACTGTGTG  
1090 1110 1130

FIG. 9D

1 50  
PDGF-A MRTWACL L L L L L GCGYLAHAL AEEAIPREL IERLARSQIH SIRD LQRLL E  
PDGF-B MNR CWA LFL SLCCYLRLVS AEGDPIPEEL YEMLS DHSIR SFDDLQRLLH  
PlGF-1 ..... MPVM RLFPC FLQ LLAGLAL  
PlGF-2 ..... MPVM RLFPC FLQ LLAGLAL  
VEGF121 ..... M NFLLS WVH WSLALLLYLH  
VEGF165 ..... M NFLLS WVH WSLALLLYLH  
VEGF189 ..... M NFLLS WVH WSLALLLYLH  
VEGF206 ..... M NFLLS WVH WSLALLLYLH  
FLT4-L ..... M TVLYPEYWK M YKCLRKG G

51 100  
PDGF-A IDSVGAEDAL ETS LRAHGSH AINHVP EKR P VPIRRKR SI ..... EEAIP  
PDGF-B GDP GEEDGA ELD LNMTRSH SGGELES LARGRRSLG SLTIAEPAMI  
PlGF-1 PAVPPQQW ..... ALSAG NGSSEVEVVP FQE VWGR  
PlGF-2 PAVPPQQW ..... ALSAG NGSSEVEVVP FQE VWGR  
VEGF121 HAKWSQAA ..... PMAEG GQNHHEVVK FMD VYQR  
VEGF165 HAKWSQAA ..... PMAEG GQNHHEVVK FMD VYQR  
VEGF189 HAKWSQAA ..... PMAEG GQNHHEVVK FMD VYQR  
VEGF206 HAKWSQAA ..... PMAEG GQNHHEVVK FMD VYQR  
FLT4-L QHNREQANLN SRTEETIKFA AAHYNTEILK SIDNEWRK

FIG. 10A

150  
101  
PDGF-A AVCKTRTVIY EIPRSQVDPT SANFLIWPPC VEVKRTGCC NTSSVKCQPS  
PDGF-B AECKTRTEVF EISRRLLDRT NANFLVWPPC VEVQRCGCGC NNRNVQCRPT  
P1GF-1 SYCRALERLV DVVSEYPS EVEHMFSPSC VSLLRCTGCC GDENLHCVPV  
P1GF-2 SYCRALERLV DVVSEYPS EVEHMFSPSC VSLLRCTGCC GDENLHCVPV  
VEGF121 SYCHPIETLV DIFQEYPD EIEYIFKPSC VPLMRGCGCC NDEGLECVPT  
VEGF165 SYCHPIETLV DIFQEYPD EIEYIFKPSC VPLMRGCGCC NDEGLECVPT  
VEGF189 SYCHPIETLV DIFQEYPD EIEYIFKPSC VPLMRGCGCC NDEGLECVPT  
VEGF206 SYCHPIETLV DIFQEYPD EIEYIFKPSC VPLMRGCGCC NDEGLECVPT  
FLT4-L TQCMPREVCI DVGKEFGV ATNTFFKPPC VSVYRCGGCC NSEGLQCMNT

200  
151  
PDGF-A RVHHRSVKVA KVEYVRKKPK LKEVQVRLEE HLEACAT . . . . . SN  
PDGF-B QVQLRPVQVR KIEIVRKKPI FKATVTLED HLACKCETVA AARPVTRSPG  
P1GF-1 ETANVTMQLL KIRSG . . . . . SYVELTFSQ HVRCECRPLR EKMKPER . . .  
P1GF-2 ETANVTMQLL KIRSG . . . . . SYVELTFSQ HVRCECRPLR EKMKPER . . .  
VEGF121 EESNITMQIM RIKPH . . . . . QGQ HIGEMSFLQ HNKCECRPKK DRARQEKD .  
VEGF165 EESNITMQIM RIKPH . . . . . QGQ HIGEMSFLQ HNKCECRPKK DRARQEN . . .  
VEGF189 EESNITMQIM RIKPH . . . . . QGQ HIGEMSFLQ HNKCECRPKK DRARQEKKS .  
VEGF206 EESNITMQIM RIKPH . . . . . QGQ HIGEMSFLQ HNKCECRPKK DRARQEKKS .  
FLT4-L STSYLSKTLF EITVPLSQGP KPTVISFAN HTSCRCMSKL DVYRQVHSII

FIG. 10B

201 250  
PDGF-A LNPDHREET DVR .....  
PDGF-B GSQEQRAKTP QTRVTIRTVR VRRPPKKGHR KFKHTHDKTA LKETLGA...  
PlGF-1 ..... CGDAVPR R .....  
PlGF-2 ..... PKGRGK RRREKQRPTD CHLCGDAVPR R .....  
VEGF121 ..... KPRR .....  
VEGF165 .....  
VEGF189 ..... VRGKGK GQKRKRKKSR YKWSV ..... P  
VEGF206 ..... VRGKGK GQKRKRKKSR YKWSVYVGA RCC ..... L MPWSLPGPHP  
FLT4-L RRSPLATLPQ CQAANKTCPT NYMWNHICR CLAQEDFMFS SDAGDDSTDG

251 300  
PDGF-A .....  
PDGF-B .....  
PlGF-1 .....  
PlGF-2 .....  
VEGF121 .....  
VEGF165 CGPCSERRKH LRVQDPQTCK CSCKNTDSRC KARQLELNER TCRCDKPRR.  
VEGF189 CGPCSERRKH LRVQDPQTCK CSCKNTDSRC KARQLELNER TCRCDKPRR.  
VEGF206 CGPCSERRKH LRVQDPQTCK CSCKNTDSRC KARQLELNER TCRCDKPRR.  
FLT4-L FHDICGPNKE LDEETCQCVC RAGLRPASCG PHKELDRNSC QCVCKNKLFP

FIG. 10C



301 350  
PDGF-A .....  
PDGF-B .....  
PlGF-1 .....  
PlGF-2 .....  
VEGF121 .....  
VEGF165 .....  
VEGF189 .....  
VEGF206 .....  
FLT4-L SQCGANREFD ENTQCQCVCKR TCPRNQPLNP GKCACECTES PQCLLKGGK

351 395  
PDGF-A .....  
PDGF-B .....  
PlGF-1 .....  
PlGF-2 .....  
VEGF121 .....  
VEGF165 .....  
VEGF189 .....  
VEGF206 .....  
FLT4-L FHHQTCSCYR RPCTNRQKAC EPGFSYSEEV CRCVPSYWKR PQMS

FIG. 10D

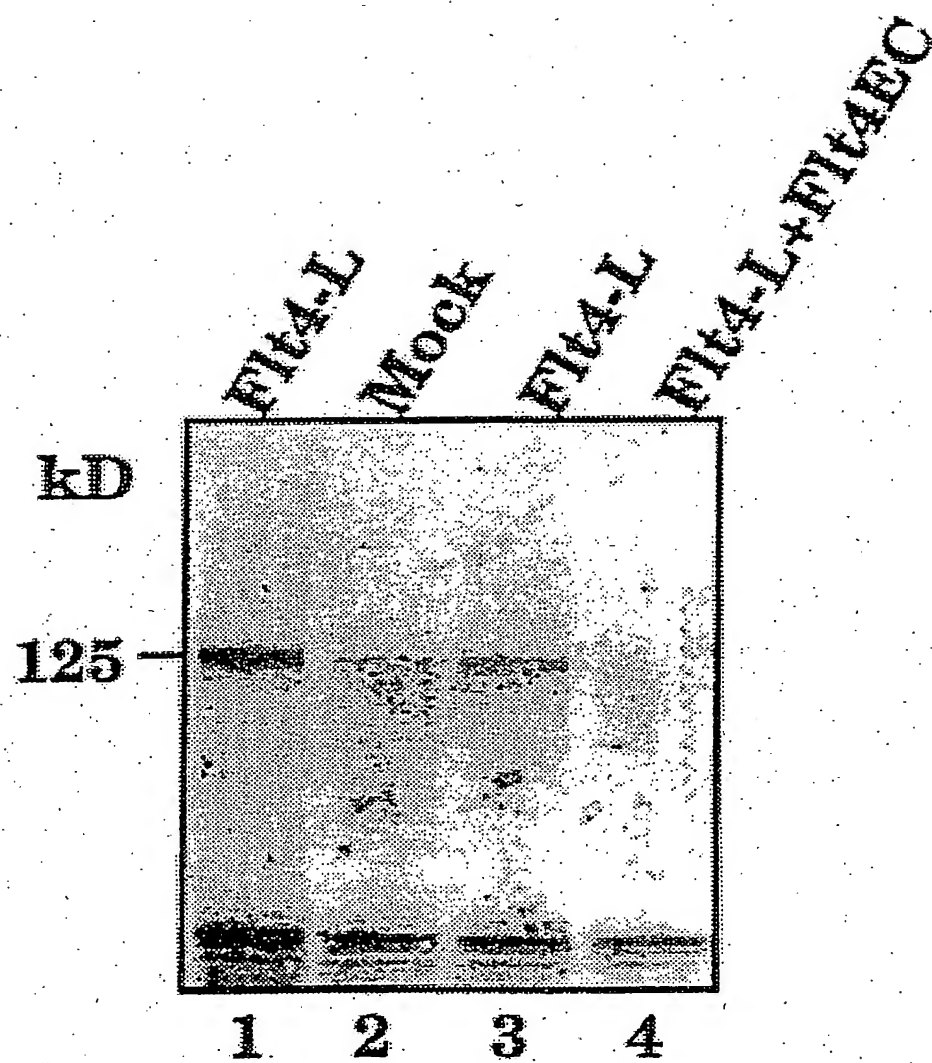


FIG. 11

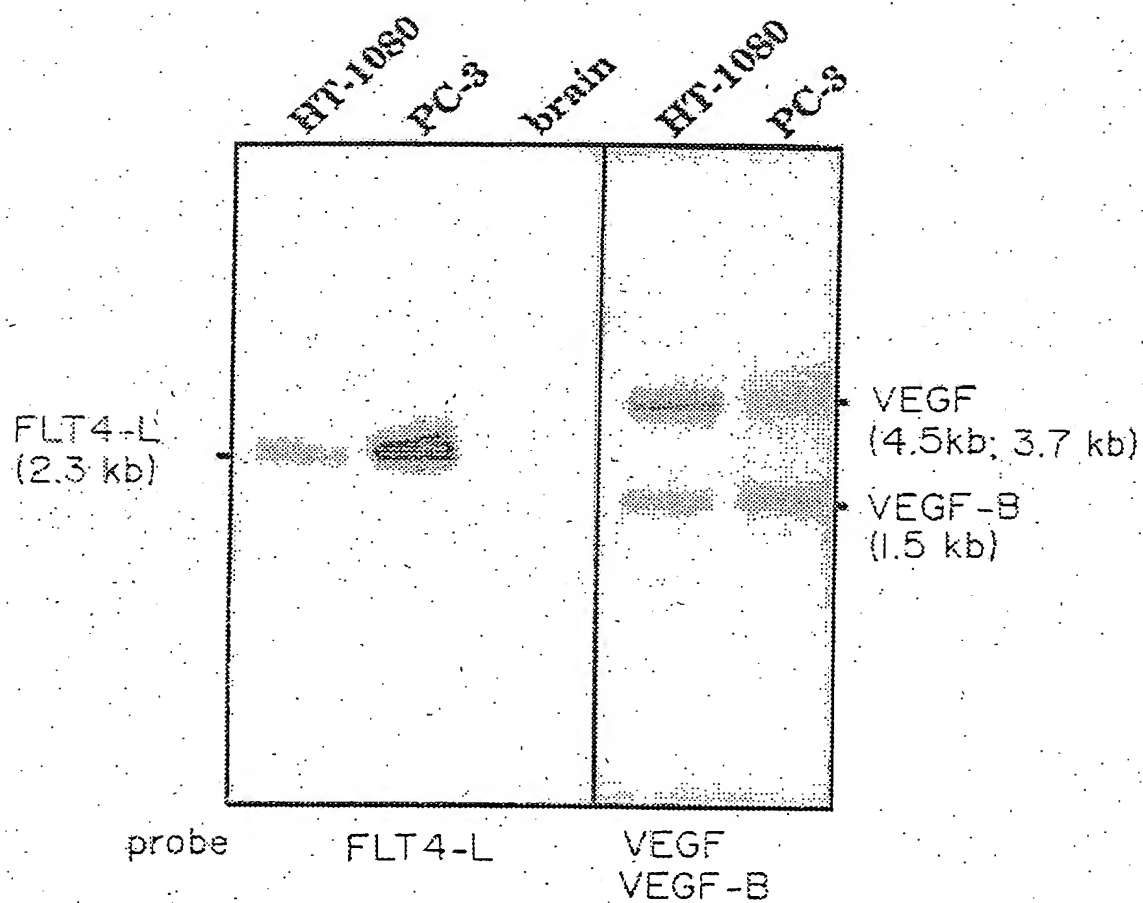


FIG. 12

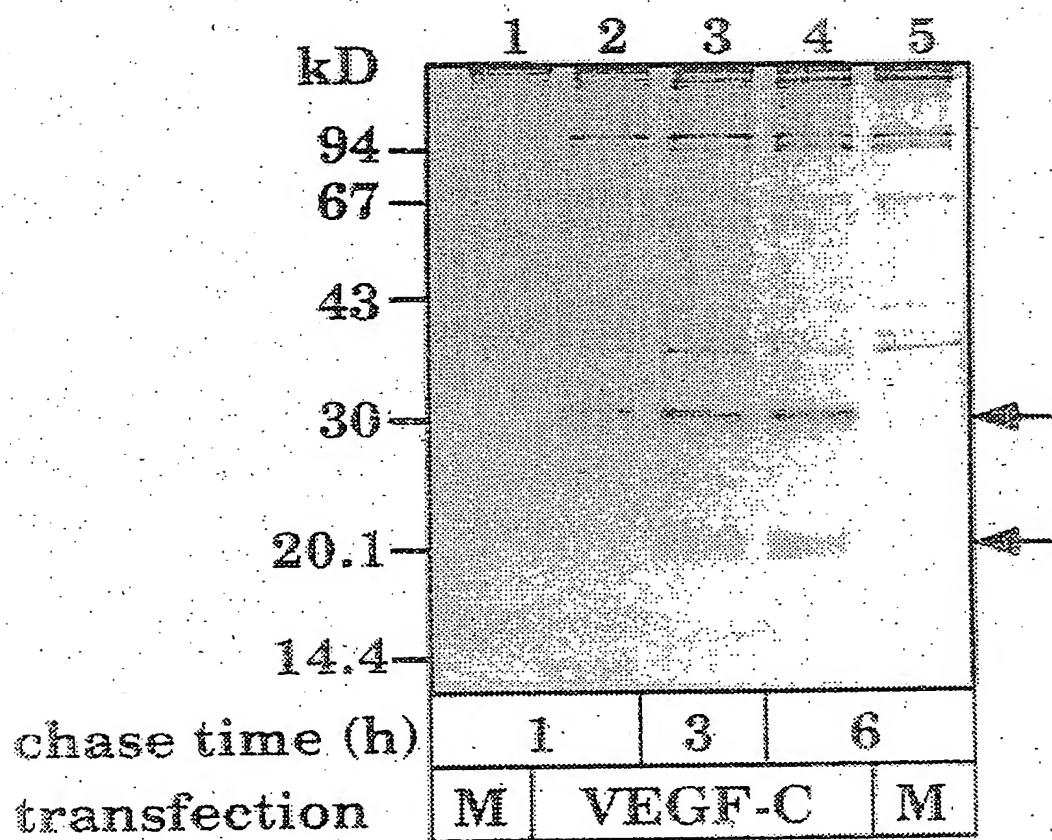


FIG. 13A

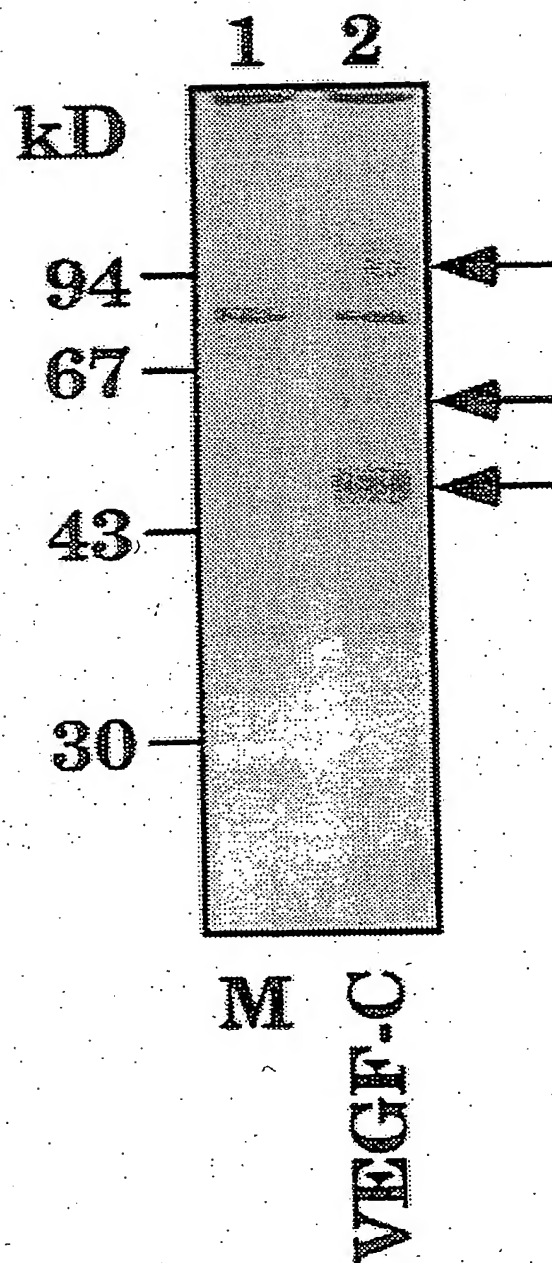


FIG. 13B

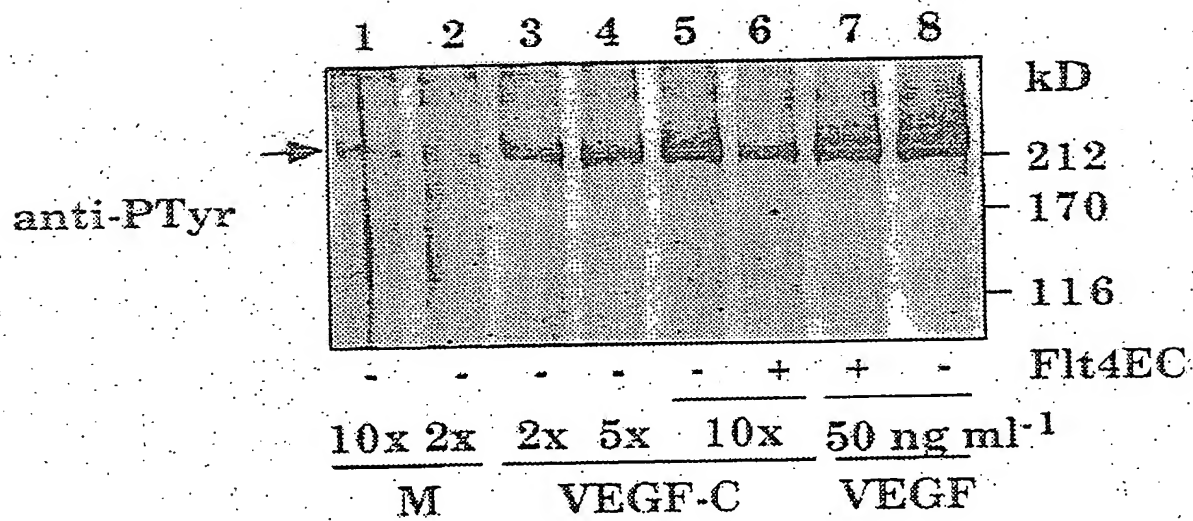


FIG. 14A

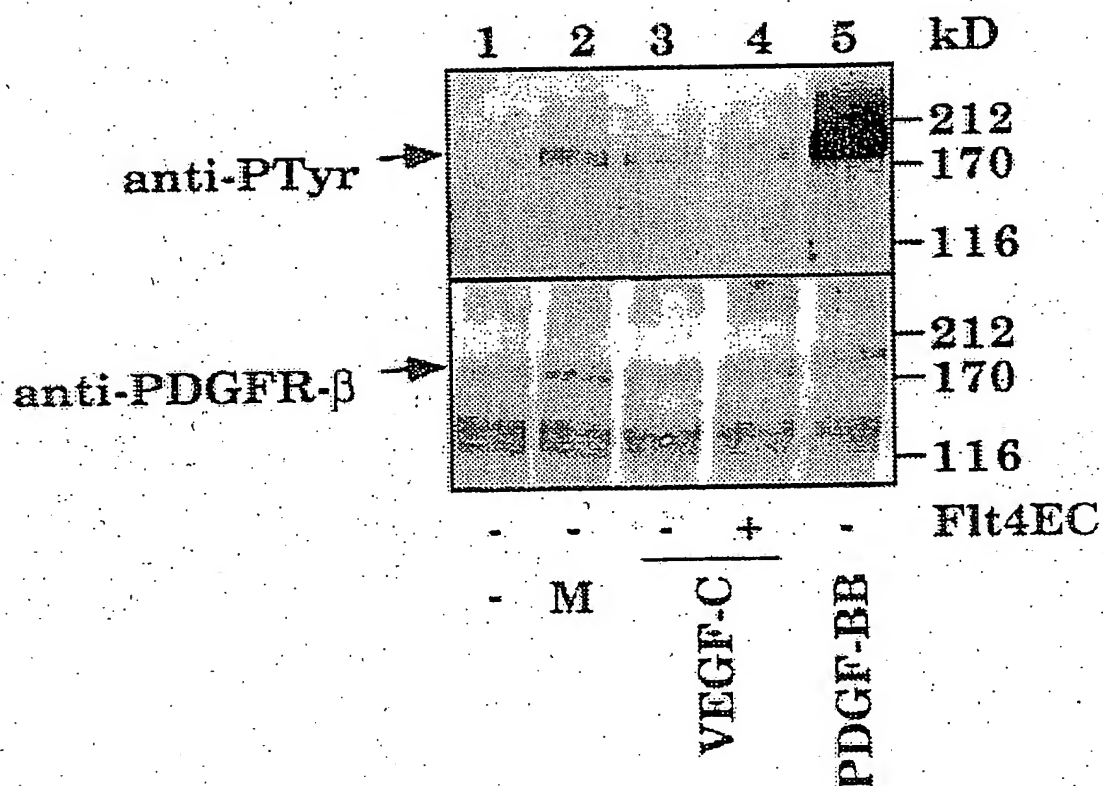


FIG. 14B

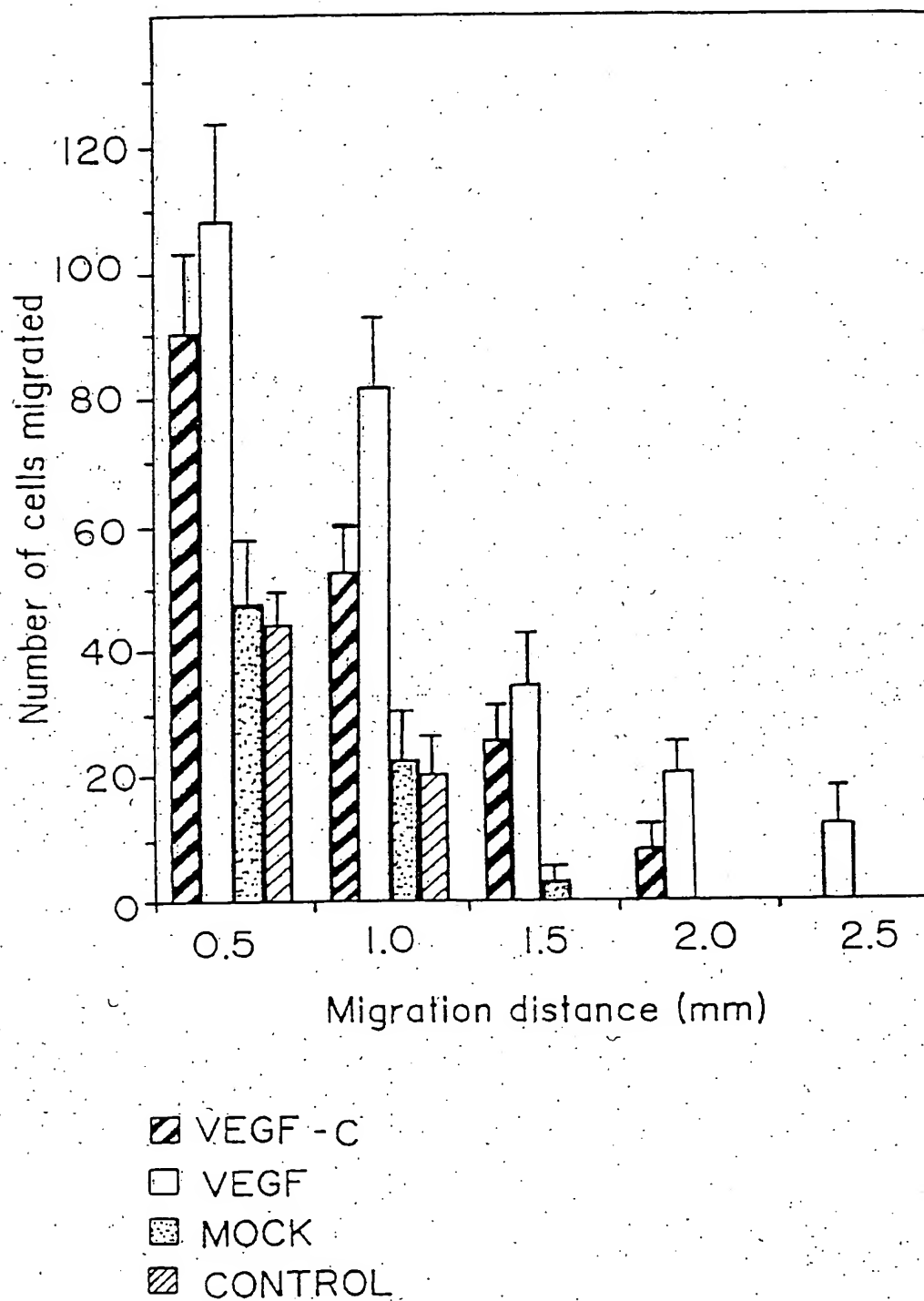


FIG. 15A



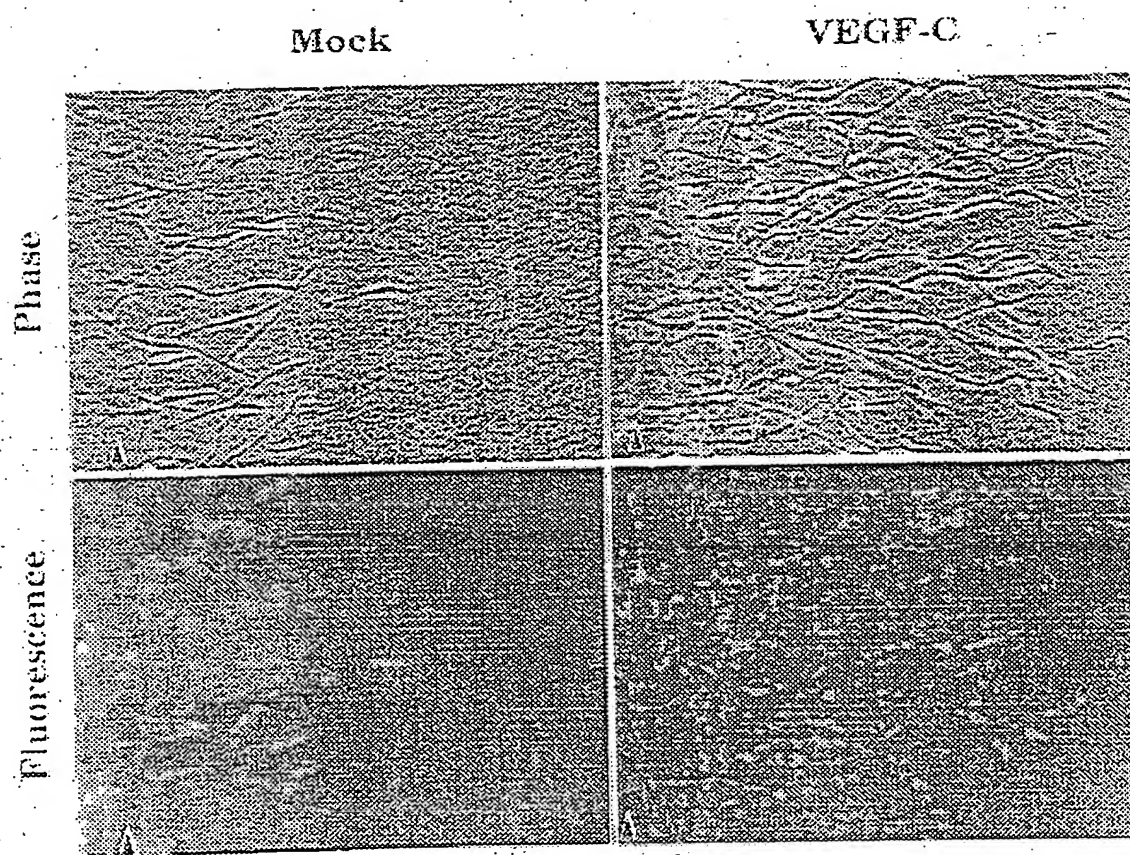


FIG. 15B

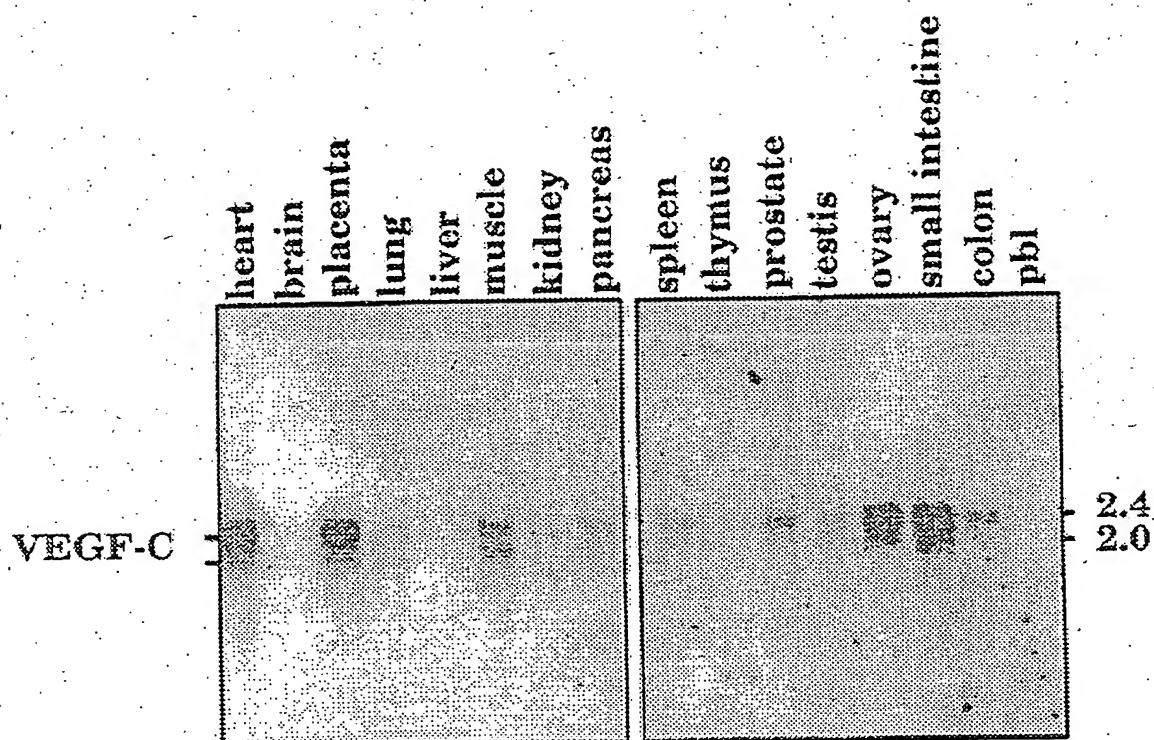


FIG. 16A

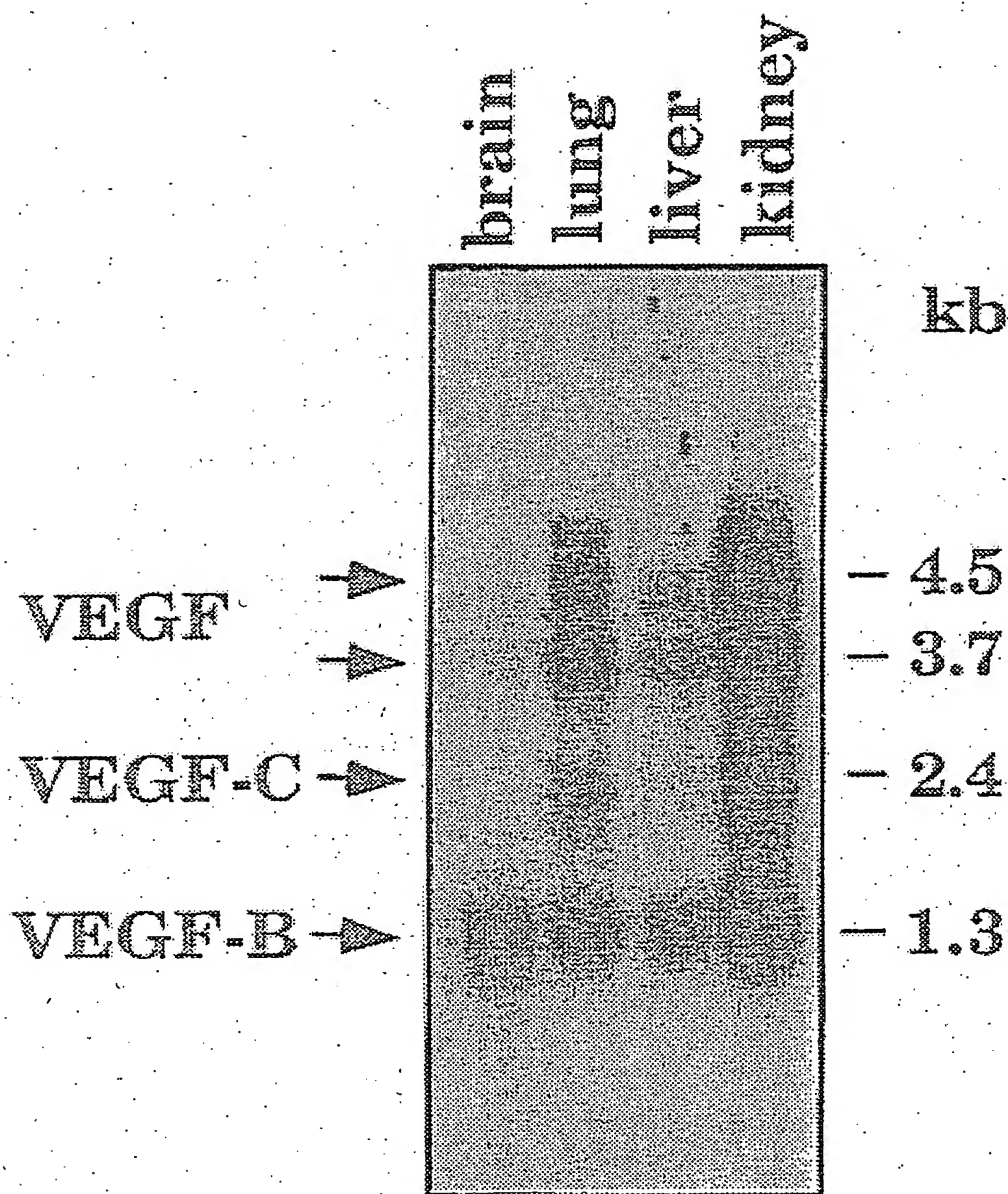


FIG. 16B

Chr 4



| VEGF-C,q34

FIG. 17

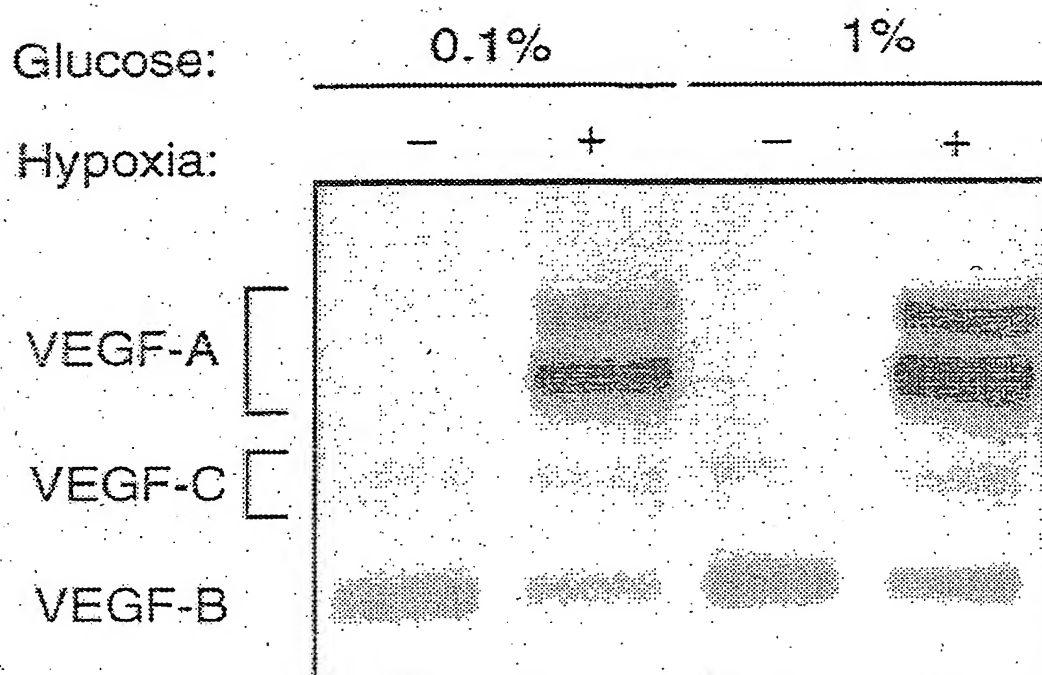


FIG. 18

## RECEPTOR LIGAND

This is a continuation-in-part of U.S. patent application Ser. No. 08/510,133, filed Aug. 1, 1995.

## FIELD OF THE INVENTION

The present invention generally relates to the field of genetic engineering and more particularly to growth factors for endothelial cells and growth factor genes.

## BACKGROUND OF THE INVENTION

Developmental growth, the remodelling and regeneration of adult tissues, as well as solid tumor growth, can only occur when accompanied by blood vessel formation. Angioblasts and hematopoietic precursor cells differentiate from the mesoderm and form the blood islands of the yolk sac and the primary vascular system of the embryo. The development of blood vessels from these early (in situ) differentiating endothelial cells is termed vasculogenesis. Major embryonic blood vessels are believed to arise via vasculogenesis, whereas the formation of the rest of the vascular tree is thought to occur as a result of vascular sprouting from pre-existing vessels, a process called angiogenesis, Risau, et al., *Devel. Biol.*, 125:441-450 (1988).

Endothelial cells give rise to several types of functionally and morphologically distinct vessels. When organs differentiate and begin to perform their specific functions, the phenotypic heterogeneity of endothelial cells increases. Upon angiogenic stimulation, endothelial cells may re-enter the cell cycle, migrate, withdraw from the cell cycle and subsequently differentiate again to form new vessels that are functionally adapted to their tissue environment. Endothelial cells undergoing angiogenesis degrade the underlying basement membrane and migrate, forming capillary sprouts that project into the perivascular stroma. Ausprunk, et al., *Microvasc. Rev.*, 14:51-65 (1977). Angiogenesis during tissue development and regeneration depends on the tightly controlled processes of endothelial cell proliferation, migration, differentiation, and survival. Dysfunction of the endothelial cell regulatory system is a key feature of many diseases. Most significantly, tumor growth and metastasis have been shown to be angiogenesis dependent. Folkman, et al., *J. Biol. Chem.*, 267: 10931-10934 (1992).

Key signals regulating cell growth and differentiation are mediated by polypeptide growth factors and their transmembrane receptors, many of which are tyrosine kinases. Autophosphorylated peptides within the tyrosine kinase insert and carboxyl-terminal sequences of activated receptors are commonly recognized by kinase substrates involved in signal transduction for the readjustment of gene expression in responding cells. Several families of receptor tyrosine kinases have been characterized. Van der Geer, et al., *Ann. Rev. Cell Biol.*, 10:251-337 (1994). The major growth factors and receptors transducing angiogenic stimuli are schematically shown in FIG. 1.

Fibroblast growth factors are also known to be involved in the regulation of angiogenesis. They have been shown to be mitogenic and chemotactic for cultured endothelial cells. Fibroblast growth factors also stimulate the production of proteases, such as collagenases and plasminogen activators, and induce tube formation by endothelial cells. Saksela, et al., *Ann. Rev. Cell Biol.*, 4:93-126 (1988). There are two general classes of fibroblast growth factors, FGF-1 and FGF-2, both of which lack conventional signal peptides. Both types have an affinity for heparin and FGF-2 is bound

to heparin sulfate proteoglycans in the subendothelial extracellular matrix from which it may be released after injury. Heparin potentiates the stimulation of endothelial cell proliferation by angiogenic FGFs, both by protecting against denaturation and degradation and dimerizing the FGFs. Cultured endothelial cells express the FGF-1 receptor but no significant levels of other high-affinity fibroblast growth factor receptors.

Among other ligands for receptor tyrosine kinases, the platelet derived growth factor, PDGF-BB, has been shown to be weakly angiogenic in the chick chorioallantoic membrane. Risau, et al., *Growth Factors*, 7:261-266 (1992). Transforming growth factor  $\alpha$  (TGF $\alpha$ ) is an angiogenic factor secreted by several tumor cell types and by macrophages. Hepatocyte growth factor (HGF), the ligand of the c-met proto-oncogene-encoded receptor, also is strongly angiogenic.

Recent evidence shows that there are endothelial cell specific growth factors and receptors that may be primarily responsible for the stimulation of endothelial cell growth, differentiation and certain differentiated functions. The best studied of these is vascular endothelial growth factor (VEGF), a member of the PDGF family. Vascular endothelial growth factor is a dimeric glycoprotein of disulfide-linked 23 kDa subunits. Other reported effects of VEGF include the mobilization of intracellular calcium, the induction of plasminogen activator and plasminogen activator inhibitor-1 synthesis, stimulation of hexose transport in endothelial cells, and promotion of monocyte migration in vitro. Four VEGF isoforms, encoded by distinct mRNA splice variants, appear to be equally capable of stimulating mitogenesis in endothelial cells. However, each isoform has a different affinity for cell surface proteoglycans, which behave as low affinity receptors for VEGF. The 121 and 165 amino acid isoforms of VEGF are secreted in a soluble form, whereas the isoforms of 189 and 206 amino acid residues remain cell surface associated and have a strong affinity for heparin.

VEGF was originally purified from several sources on the basis of its mitogenic activity toward endothelial cells, and also by its ability to induce microvascular permeability, hence it is also called vascular permeability factor (VPF). VEGF produces signals through two receptor tyrosine kinases, VEGFR-1 (FLT-1) and VEGFR-2 (KDR/Flk-1), which are expressed specifically on endothelial cells. The VEGF-related placenta growth factor (PlGF) was recently shown to bind to VEGFR-1 with high affinity. PlGF was able to enhance the growth factor activity of VEGF, but it did not stimulate endothelial cells on its own. Naturally occurring VEGF/PlGF heterodimers were nearly as potent mitogens as VEGF homodimers for endothelial cells.

The pattern of VEGF expression suggests its involvement in the development and maintenance of the normal vascular system and in tumor angiogenesis. During murine development, the entire 7.5 day post-coital (p.c.) endoderm expresses VEGF and the ventricular neuroectoderm produces VEGF at the capillary ingrowth stage. See Breier, et al., *Development*, 114:521-523 (1992). On day two of quail development, the vascularized area of the yolk sac as well as the whole embryo show expression of VEGF. In addition, epithelial cells next to fenestrated endothelia in adult mice show persistent VEGF expression, suggesting a role in the maintenance of this specific endothelial phenotype and function.

Two high affinity receptors for VEGF have been characterized. These are VEGFR-1/Flt-1 (fms-like tyrosine kinase-

1) and VEGFR-2/Kdr/Flk-1 (kinase insert domain containing receptor/fetal liver kinase-1). Those receptors are classified in the PDGF-receptor family, but they have seven rather than five immunoglobulin-like loops in their extracellular domain and they possess a longer kinase insert than normally observed in this family. The expression of VEGF receptors occurs mainly in vascular endothelial cells, although some may be present on monocytes and melanoma cells. Only endothelial cells have been reported to proliferate in response to VEGF, and endothelial cells from different sources show different responses. Thus, the signals mediated through VEGFR-1 and VEGFR-2 appear to be cell type specific.

The Flt4 receptor tyrosine kinase (VEGFR-3) is closely related in structure to the products of the VEGFR-1 and VEGFR-2 genes. Despite this similarity, the mature form of Flt4 differs from the VEGF receptors in that it is proteolytically cleaved in the extracellular domain into two disulfide-linked polypeptides. Pajusola et al., *Cancer Res.*, 52:5738-5743 (1992). The 4.5 and 5.8 kb Flt4 mRNAs encode polypeptides which differ in their C-termini due to the use of alternative 3' exons. The VEGFs do not show specific binding to Flt4 or induce its autophosphorylation.

Expression of Flt4 appears to be more restricted than expression of VEGFR-1 or VEGFR-2. The expression of Flt4 first becomes detectable by in situ hybridization in the angioblasts of head mesenchyme, the cardinal vein, and extraembryonically in the allantois of 8.5 day p.c. mouse embryos. In 12.5 day p.c. embryos the Flt4 signal is observed in developing venous and presumptive lymphatic endothelia, but arterial endothelia appear negative. During later stages of development, Flt4 mRNA becomes restricted to developing lymphatic vessels. Only the lymphatic endothelia and some high endothelial venules express Flt4 mRNA in adult human tissues and increased expression occurs in lymphatic sinuses in metastatic lymph nodes and in lymphangioma. These results support the theory of the venous origin of lymphatic vessels.

Five endothelial cell specific receptor tyrosine kinases, Flt-1 (VEGFR-1), KDR/Flk-1 (VEGFR-2), Flt4, Tie and Tek/Tie-2 have so far been described, which possess the intrinsic tyrosine kinase activity essential for signal transduction. Targeted mutations inactivating Flt-1, Flk-1, Tie and Tek in mouse embryos have indicated their essential and specific roles in vasculogenesis and angiogenesis at the molecular level. VEGFR-1 and VEGFR-2 bind VEGF with high affinity (Kd 16 pM and 760 pM, respectively) and VEGFR-1 also binds the related placenta growth factor (PIGF; Kd about 200 pM), while the ligands for Tie, Tek, and Flt4 have not yet been reported.

#### SUMMARY OF THE INVENTION

The present invention provides a ligand for the Flt4 receptor tyrosine kinase. Thus, the invention provides a purified and isolated polypeptide which specifically binds to the Flt4 receptor tyrosine kinase. In a preferred embodiment, the ligand comprises a fragment of the amino acid sequence shown in SEQ ID NO: 33 which specifically binds to the Flt4 receptor tyrosine kinase.

The present invention also provides a precursor of an Flt4 ligand, wherein the precursor comprises the amino acid sequence shown in SEQ ID NO: 33. Thus, the invention includes a purified and isolated polypeptide having the amino acid sequence shown in SEQ ID NO: 33.

A putative 33 amino acid signal peptide has been identified in the amino acid sequence shown in SEQ ID NO: 33.

Thus, in a related aspect, the invention includes a purified and isolated polypeptide comprising amino acids 1-317 of SEQ ID NO: 33. The Flt4 ligand precursor is proteolytically cleaved upon expression to produce an approximately 23 kD peptide which is the Flt4 ligand (herein designated VEGF-C). Thus, the invention includes a polypeptide having an amino acid sequence comprising a portion of SEQ ID NO: 33, the portion encoding a fragment capable of specifically binding to Flt4. A preferred fragment has a molecular weight of about 23 kDa as assessed by SDS-PAGE under reducing conditions. In a preferred embodiment of the invention, an Flt4 ligand is provided which is the cleavage product of the precursor peptide shown in SEQ ID NO: 33 and which has a molecular weight of approximately 23 kD under reducing conditions.

Evidence suggests that the amino acids essential for retaining Flt4 ligand activity are contained within approximately amino acids 1-120 of SEQ ID NO: 33, and that the proteolytic cleavage to produce a mature, naturally-occurring Flt4 ligand occurs within approximately amino acids 1-180 of SEQ ID NO: 33. Accordingly, preferred polypeptides of the invention include polypeptides comprising amino acids 1-120, 1-121, 1-122, 1-123, 1-124, 1-178, 1-179, and 1-180 of SEQ ID NO: 33, wherein said polypeptides specifically bind to an Flt4 receptor tyrosine kinase. A preferred Flt4 ligand comprises approximately amino acids 1-120 of SEQ ID NO: 33. Another preferred polypeptide of the invention comprises approximately amino acids 1-180 of SEQ ID NO: 33.

The present invention also provides a cDNA encoding a novel polypeptide, designated VEGF-C, that is structurally homologous to VEGF. VEGF-C is a ligand for the FLT4 receptor tyrosine kinase (VEGFR-3), a receptor tyrosine kinase related to VEGFR-1 and VEGFR-2 that does not bind VEGF. VEGFR-3 is expressed in venous and lymphatic endothelia of fetal tissues and predominantly in lymphatic endothelial of adult tissues. Kaipainen et al., *Cancer Res.*, 54:6571-77 (1994); Kaipainen et al., *Proc. Natl. Acad. Sci. USA*, 92:3566-70 (1995).

Thus, in a preferred embodiment, the invention includes a purified and isolated nucleic acid (e.g., a DNA or an RNA) encoding an Flt4 ligand precursor. Due to the degeneracy of the genetic code, numerous such coding sequences are possible, each having in common the coding of the amino acid sequence shown in SEQ ID NO: 33. As set forth above, the invention includes polypeptides which comprise a portion of the amino acid sequence shown in SEQ ID NO: 33 and which bind the Flt4 receptor tyrosine kinase (herein designated VEGFR-3); the invention also is intended to include nucleic acids encoding these polypeptides. Ligand precursors according to the invention, when expressed in an appropriate host cell, produce, via cleavage, a peptide which binds specifically to the Flt4 receptor tyrosine kinase (VEGFR-3). The nucleotide sequence shown in SEQ ID NO: 33 contains a preferred nucleotide sequence encoding the Flt4 ligand (VEGF-C).

The present invention also provides a cell line which produces an Flt4 ligand. The ligand may be purified and isolated directly from the cell culture medium. Also provided are vectors comprising a DNA encoding the Flt4 ligand, and host cells comprising the vectors. Preferred vectors of the invention are capable of expressing the Flt4 ligand under the control of appropriate promoters and other control sequences. A preferred vector of the invention is plasmid pFLT4-L, having ATCC accession no. 97231.

The invention further includes a method of making polypeptides of the invention. In a preferred method, a



nucleic acid or vector of the invention is expressed in a host cell, and a polypeptide of the invention is purified from the host cell or the host cell growth medium.

In another aspect, the invention includes an antibody which is specifically reactive with polypeptides of the invention. Antibodies, both monoclonal and polyclonal, may be made against a ligand of the invention according to standard techniques in the art. Such antibodies may be used in diagnostic applications to monitor angiogenesis, vascularization, lymphatic vessels and their disease states, wound healing, or certain hematopoietic or leukemia cells, or they may be used to block or activate the Flt4 receptor.

Ligands according to the invention may be labeled with a detectable label and used to identify their corresponding receptors *in situ*. Labeled Flt4 ligand and anti-Flt4 ligand antibodies may be used as imaging agents in the detection of lymphatic vessels, high endothelial venules, and Flt4 receptors expressed in histochemical tissue sections. The ligand or antibody may be covalently or non-covalently coupled to a suitable supermagnetic, paramagnetic, electron dense, echogenic, or radioactive agent for imaging. Other, non-radioactive labels, such as biotin and avidin, may also be used.

The present invention also provides diagnostic and clinical applications for claimed ligands. In a preferred embodiment, Flt4 ligands or precursors are used to accelerate angiogenesis, e.g., during wound healing, or to promote the endothelial functions of lymphatic vessels. Ligands may be applied in any suitable manner using an appropriate pharmaceutically-acceptable vehicle. Ligands also may be used to quantify future metastatic risk by assaying biopsy material for the presence of active receptors or ligands in a binding assay or kit using detectably-labeled ligand. An Flt4 ligand according to the invention also may be used to promote re-growth or permeability of lymphatic vessels in, for example, organ transplant patients. Ligands according to the invention also may be used to treat or prevent inflammation, edema, aplasia of the lymphatic vessels, lymphatic obstruction, elephantiasis, and Milroy's disease. Finally, Flt4 ligands may be used to stimulate lymphocyte production and maturation, and to promote or inhibit trafficking of leukocytes between tissues and lymphatic vessels or to affect migration in and out of the thymus.

Inhibitors of the Flt4 ligand may be used to control endothelial cell proliferation and lymphangiomas. For example, such inhibitors may be used to arrest metastatic growth or spread, or to control other aspects of endothelial cell expression and growth. Inhibitors include antibodies, antisense oligonucleotides, and peptides which block the Flt4 receptor, all of which are intended as aspects of the invention.

#### BRIEF DESCRIPTION OF THE DRAWING

FIG. 1 is a schematic diagram showing major endothelial cell receptor tyrosine kinases and growth factors involved in vasculogenesis and angiogenesis.

FIGS. 2A and 2B schematically depict the construction of the pLTRFlt4l expression vector.

FIG. 3 schematically depicts the construction of the baculovirus vector encoding a secreted soluble Flt4 extracellular domain (Flt4EC).

FIG. 4 shows results of stimulation of Flt4 autophosphorylation by conditioned medium from PC-3 cell cultures.

FIGS. 5A, 5B, and 5C show that the major tyrosyl phosphorylated polypeptide of Flt4-transfected cells stimu-

lated with PC-3 conditioned medium is the 125 kD Flt4 polypeptide (VEGFR-3).

FIG. 6 shows Western analysis of the Flt4 ligand activity isolated from PC-3 conditioned medium.

FIG. 7 shows results of gel electrophoresis of chromatographic fractions from the affinity purification of Flt4 ligand (VEGF-C) isolated from PC-3 conditioned medium.

FIG. 8 shows results of Western analysis of Flt4 autophosphorylation induced by either the Flt4 ligand (VEGF-C), VEGF, or PIGF.

FIG. 9A schematically depicts the cloning and analysis of the Flt4 ligand, VEGF-C. The VEGF-C coding sequence (shaded boxes) and signal sequence (ss) are depicted between 5' and 3' untranslated (ut) nucleic acid regions.

FIGS. 9B-D show the nucleotide and deduced amino acid sequence of the coding portion of Flt4 ligand cDNA. The cleavage site for the putative signal peptide is indicated with a shaded triangle.

FIGS. 10A-D show a comparison of the deduced amino acid sequences of PDGF-A (SEQ ID NO: 36); PDGF-B (SEQ ID NO: 37); two PIGF isoforms (SEQ ID NOs: 38 and 39); four VEGF isoforms (SEQ ID NOs: 40-43); and Flt4 ligand (VEGF-C) (SEQ ID NO: 33).

FIG. 11 shows the stimulation of autophosphorylation of the Flt4 receptor by conditioned medium from cells transfected with the Flt4-L (VEGF-C) expression vector.

FIG. 12 shows Northern blotting analysis of Flt4-L (VEGF-C) mRNA in tumor cell lines and in brain tissue.

FIG. 13A is an autoradiograph showing recombinant VEGF-C isolated following a pulse-chase experiment and electrophoresed via SDS-PAGE under reducing conditions.

FIG. 13B is a photograph of polyacrylamide gel showing that recombinant VEGF-C forms are disulfide-linked in nonreducing conditions.

FIGS. 14A and 14B depict Western blots showing that VEGF-C stimulates autophosphorylation of VEGFR-2 (KDR) but has no effect on PDGFR- $\beta$  phosphorylation.

FIGS. 15A and 15B show that VEGF-C stimulates endothelial cell migration in a three-dimensional collagen gel assay.

FIG. 16A shows the expression of VEGF-C mRNA in human adult tissues.

FIG. 16B shows the expression of VEGF, VEGF-B, and VEGF-C in selected human fetal tissues.

FIG. 17 schematically depicts the chromosomal localization of the VEGF-C gene.

FIG. 18 is a Northern blot hybridization study showing the effects of hypoxia on the mRNA expression of VEGF-A, VEGF-B and VEGF-C.

#### DETAILED DESCRIPTION OF THE INVENTION

Described herein is the isolation of a novel vascular endothelial growth factor and the cloning of a DNA encoding this growth factor from a cDNA library prepared from the human prostatic adenocarcinoma cell line PC-3. The isolated cDNA encodes a protein which is proteolytically processed and secreted to cell culture medium. The secreted protein, designated VEGF-C, binds to the extracellular domain of Flt4 (designated VEGFR-3) and induces tyrosine autophosphorylation of Flt4 and VEGFR-2. VEGF-C also stimulates the migration of endothelial cells in collagen gel.

The present invention also is directed to novel growth factors which are ligands for the Flt4 receptor tyrosine



kinase (VEGFR-3). Ligands of the invention are members of a family of platelet-derived growth factors/vascular endothelial growth factors which promote mitosis and proliferation of vascular endothelial cells and/or mesodermal cells. Ligands recognizing the Flt4 receptor tyrosine kinase were purified from a PC-3 prostatic adenocarcinoma cell line (ATCC CRL1435). When applied to a population of cells expressing the Flt4 receptor, ligands of the invention stimulate autophosphorylation, resulting in receptor activation. The invention also provides inhibitors of the Flt4 receptor, including antibodies directed against the ligand. A ligand according to the invention may be coexpressed as a larger precursor which is cleaved to produce the ligand. A coexpressed region in some cases results from alternative splicing of RNA of the ligand gene. Such a co-expressed region may be a function of the particular expression system used to obtain the ligand. The skilled artisan understands that in recombinant production of proteins, additional sequence may be expressed along with a functional peptide depending upon the particular recombinant construct used to express the protein, and subsequently removed to obtain the desired ligand. In some cases the recombinant ligand can be made lacking certain residues of the endogenous/natural ligand. Moreover, it is well-known in that conservative replacements may be made in a protein which do not alter the function of the protein. Accordingly, it is anticipated that such alterations are within the scope of the claims. It is intended that the precursor sequence shown in SEQ ID NO: 33 is capable of stimulating the Flt4 ligand without any further processing in a manner similar to that in which VEGF stimulates its receptor in its unprocessed form.

Results reported herein show that VEGFR-3 transmits signals for a novel growth factor. This conclusion is based on the specific binding of VEGF-C to recombinant Flt4EC (Flt4 extracellular domain) protein and the induction of VEGFR-3 autophosphorylation by medium from VEGF-C transfected cells. In contrast, VEGF and PlGF did not show specific binding to VEGFR-3 or induce its autophosphorylation.

A major part of the difference in the observed molecular mass of the purified and recombinant VEGF-C and the deduced molecular mass of the VEGF-C encoded by the VEGF-C open reading frame (ORF) may be due to proteolytic removal of sequences in the carboxyl terminal region of the latter. Proteolytic processing of the VEGF-C precursor may occur at more than one cleavage site because the 32 kD molecular mass of the recombinant secreted ligand was also less than the deduced molecular mass of VEGF-C ORF without the signal peptide. By extrapolation from studies of the structure of PDGF (Heldin, et al., *Growth Factors*, 8:245-52 (1993)), one can speculate that the region critical for receptor binding and activation by VEGF-C is contained within the amino-terminal first 180 or so amino acid residues of the secreted VEGF-C protein lacking the signal sequence. In fact, the region critical for receptor binding and activation by VEGF-C is believed to be contained within the first approximately 120 amino acid residues of the secreted VEGF-C protein lacking the signal sequence. Thus, the 23 kD polypeptide binding VEGFR-3 is likely to represent the VEGF-homologous domain. After biosynthesis, the nascent VEGF-C polypeptide may be glycosylated at three putative N-linked glycosylation sites identified in the deduced VEGF-C amino acid sequence.

The carboxyl terminal amino acid sequences, which increase the length of the VEGF-C polypeptide in comparison with other ligands of this family, show a pattern of spacing of cysteine residues reminiscent of the Balbiani ring 3 protein (BR3P) sequence (Dignam and Case, *Gene*,

88:133-40 (1990); Paulsson, et al., *J. Mol. Biol.*, 211:331-49 (1990)). This novel C-terminal silk protein-like structural motif of VEGF-C may fold into an independent domain, which, on the basis of the considerations above, is at least partially cleaved off after biosynthesis. Interestingly, at least one cysteine motif of the BR3P type is also found in the carboxyl terminus of VEGF. In our experiments both the putative precursor and cleaved ligand were detected in the cell culture media, although processing was apparently cell-associated on the basis of the pulse-chase experiments. The determination of the amino terminal sequence of the isolated carboxyl terminal fragment will allow the identification of the proteolytic processing site. The generation of antibodies against different parts of the VEGF-C molecule will allow the exact determination of the precursor-product relationship and ratio, their cellular distribution, and the kinetics of processing and secretion.

VEGF-C has a conserved pattern of eight cysteine residues, which may participate in the formation of intra- and interchain disulfide bonds, creating an antiparallel dimeric biologically active molecule, similar to PDGF. Mutational analysis of the cysteine residues involved in the interchain disulfide bridges has shown that, in contrast to PDGF, VEGF dimers need to be held together by these covalent interactions in order to maintain biological activity. Disulfide linking of the VEGF-C polypeptide chain was evident in the analysis of VEGF-C in nonreducing conditions.

VEGFR-3, which thus distinguishes between VEGF and VEGF-C, is closely related in structure to VEGFR-1 and VEGFR-2. Finnerty, et al., *Oncogene*, 8:2293-98 (1993); Galland, et al., *Oncogene*, 8:1233-40 (1993); Pajusola, et al., *Cancer Res.*, 52:5738-43 (1992). However, the mature form of VEGFR-3 differs from the two other VEGFRs in that it is proteolytically cleaved in the extracellular domain into two disulfide-linked polypeptides. Pajusola, et al., *Oncogene*, 9:3545-55 (1994). Another difference is that the 4.5 and 5.8 kb VEGFR-3 mRNAs encode polypeptides differing in their C-termini and apparently in their signalling properties due to the use of alternative 3' exons. Borg et al., *Oncogene*, 10:973-84 (1995); Pajusola et al., *Oncogene*, 8:2931-37 (1993).

Besides VEGFR-3, VEGFR-2 tyrosine kinase also is activated in response to VEGF-C. VEGFR-2 mediated signals cause striking changes in the morphology, actin reorganization and membrane ruffling of porcine aortic endothelial cells overexpressing this receptor. In these cells, VEGFR-2 also mediated ligand-induced chemotaxis and mitogenicity. Waltenberger et al., *J. Biol. Chem.*, 269:26988-95 (1994). Similarly, the receptor chimera CSF-1R/VEGFR-3 was mitogenic when ectopically expressed in NIH3T3 fibroblastic cells, but not in porcine aortic endothelial cells (Pajusola et al., 1994). Consistent with such results, the bovine capillary endothelial cells (BCE), which express VEGFR-2 mRNA but very little or no VEGFR-1 or VEGFR-3 mRNAs, showed enhanced migration when stimulated with VEGF-C. As shown here, light microscopy of the BCE cell cultures in collagen gel also suggested that VEGF-C stimulated the proliferation of these cells. The already existing data thus indicate that the VEGF ligands and receptors show a great specificity in their signalling, which may be cell type dependent.

The expression pattern of the VEGFR-3 (Kaipainen et al., *Proc. Natl. Acad. Sci. USA*, 92:3566-70 (1995)) suggests that VEGF-C may function in the formation of the venous and lymphatic vascular systems during embryogenesis. Constitutive expression of VEGF-C in adult tissues shown

herein further suggests that this gene product also is involved in the maintenance of the differentiated functions of the lymphatic endothelium where VEGFR-3 is expressed (Kaipainen et al., 1995). Lymphatic capillaries do not have well formed basal laminae and an interesting possibility remains that the silk-like BR3P motif is involved in producing a supramolecular structure which could regulate the availability of VEGF-C in tissues. However, as shown here, VEGF-C also activates VEGFR-2, which is abundant in proliferating endothelial cells of vascular sprouts and branching vessels of embryonic tissues, but decreased in adult tissues. Millauer et al., *Nature*, 367:576-78 (1993). These data have suggested that VEGFR-2 is a major regulator of vasculogenesis and angiogenesis. VEGF-C may thus have a unique effect in lymphatic endothelium and a more redundant function shared with VEGF in angiogenesis and possibly permeability regulation of several types of endothelia. Because VEGF-C stimulates VEGFR-2 and promotes endothelial migration, a utility for VEGF-C is suggested as an inducer of angiogenesis of blood and lymphatic vessels in wound healing, tissue transplantation, in eye diseases, in the formation of collateral vessels around arterial stenoses and into injured tissues after infarction.

Taken together, these results show an increased complexity of signalling in the vascular endothelium. They reinforce the concept that when organs differentiate and begin to perform their specific functions, the phenotypic heterogeneity of endothelial cells increases in several types of functionally and morphologically distinct vessels. However, upon suitable angiogenic stimuli, endothelial cells can re-enter the cell cycle, migrate, withdraw from the cell cycle and subsequently differentiate again to form new vessels that are functionally adapted to their tissue environment. This process of angiogenesis concurrent with tissue development and regeneration depends on the tightly controlled balance between positive and negative signals for endothelial cell proliferation, migration, differentiation and survival. Previously-identified growth factors promoting angiogenesis include the fibroblast growth factors, hepatocyte growth factor/scatter factor, PDGF and TGF- $\alpha$ . (See, e.g., Folkman, *Nature Med.* 1:27-31 (1995); Friesel and Maciag, *FASEB J.* 9:919-25 (1995); Mustonen and Alitalo, *J. Cell Biol.* 129:895-98 (1995). However, VEGF has been the only growth factor relatively specific for endothelial cells. The newly identified factors VEGF-B and VEGF-C thus increase our understanding of the complexity of the specific and redundant positive signals for endothelial cells involved in vasculogenesis, angiogenesis, permeability and perhaps also other endothelial functions.

Also described herein is the localization of the VEGF-C gene in human chromosomes by analysis of somatic cell hybrids and fluorescence in situ hybridization (FISH). Southern blotting and polymerase chain reaction analysis of somatic cell hybrids and fluorescence in situ hybridization of metaphase chromosomes was used to assess the chromosomal localization of the VEGF-C gene. The VEGF-C gene was located on chromosome 4q34, close to the human aspartylglucosaminidase gene previously mapped to 4q34-35. The VEGF-C locus in 4q34 is a candidate target for mutations leading to vascular malformations or cardiovascular diseases. Expression studies by Northern blotting and hybridization show abundant VEGF-C expression in heart and skeletal muscle; other tissues, such as lung and kidney, also express this gene. Whereas PlGF is predominantly expressed in the placenta, the expression patterns of the three VEGFs overlap in many tissues, which suggests that they may form heterodimers and interact to exert their physiological functions.

Targeted mutagenesis leading to inactivation of the VEGF receptor loci in the mouse genome has shown that VEGFR-1 is necessary for the proper organization of endothelial cells forming the vascular endothelium, while VEGFR-2 is necessary for the generation of both endothelial and hematopoietic cells. This suggests that the four genes of the VEGF family can be targets for mutations leading to vascular malformations or cardiovascular diseases.

The following Examples illustrate preferred embodiments of the invention, wherein the isolation, characterization, and function of Flt4 ligands and ligand-encoding nucleic acids according to the invention are shown.

#### EXAMPLE 1

##### Production of pLTRFlt4 Expression Vector

Construction of the LTR-Flt4 vector is schematically shown in FIGS. 2A and 2B. The full-length Flt4s cDNA (Genbank Accession No. X68203) was assembled by first subcloning the S2.5 fragment, reported in Pajusola et al., *Cancer Res.* 52:5738-5743 (1992), incorporated by reference herein, containing base pairs 56-2534 of the Flt4s into the EcoRI site of the pSP73 vector (Promega, Madison, Wis.).

Since cDNA libraries used for screening of Flt4 cDNAs did not contain its most 5' protein-coding sequences, inverse PCR was used for the amplification of the 5' end of Flt4 corresponding to the first 12 amino acid residues (MQRGAALCLRLW). Poly(A)<sup>+</sup> RNA was isolated from HEL cells and double-stranded cDNA copy was synthesized using the Amersham cDNA Synthesis System Plus kit and a gene specific primer: 5'-TGTCCTCGCTGTCCTTGCT-3' (SEQ ID NO: 1), which was located 195 bp downstream of the 5' end of clone S2.5. Double stranded cDNA was treated with T4 DNA polymerase to blunt the ends and cDNA was purified with Centricon 100 filters (Amicon Inc., Beverly, Mass.). Circularization was made in a total volume of 150  $\mu$ l. The reaction mixture contained ligation buffer, 5% PEG-8000, 1 mM DTT and 8U of T4 DNA ligase (New England Biolabs). Ligation was carried out at 16° C. for 16 hours. Fifteen  $\mu$ l of this reaction mix was used in a standard 100  $\mu$ l PCR reaction containing 100 ng of specific primers including SacI and PstI restriction sites, present in this segment of the Flt4 cDNA, and 1 unit of Taq DNA polymerase (Perkin Elmer Cetus). Two rounds of PCR were performed using 33 cycles (denaturation at 95° C. for 1 minute, annealing at 55° C. for 2 minutes and elongation at 72° C. for 4 minutes). The PCR mixture was treated sequentially with the SacI and PstI restriction enzymes and after purification with MagicPCR Preps (Promega) DNA fragments were subcloned into the pGEM3Zf(+) vector for sequencing. The sequence obtained corresponds to the 5' end of the Flt4s cDNA clone deposited in the Genbank Database as Accession No. X68203.

The sequence encoding the first 12 amino acid residues was added to the expression construct by ligating an SphI digested PCR fragment amplified using reverse transcription-PCR of poly(A)<sup>+</sup> RNA isolated from the HEL cells using the oligonucleotides 5'-ACATGCATGC-CAC-CATGCAG CGGGGCGCCG CGCTGTGCCT GCGACTGTGG CTCTGCCTGG GACTCCTGGA-3' (SEQ ID NO: 2)(forward primer, SphI site underlined, the translational start codon marked in bold follows an optimized Kozak consensus sequence Kozak, *Nucl. Acids Res.* 15: 8125-8148, 1987) and 5'-ACATGCATGC CCGCCGGT CATCC-3' (SEQ ID NO: 3) (reverse primer, SphI site underlined) to the 5' end of the S2.5 fragment, thus replacing

unique SphI fragment of the S2.5 plasmid. The resulting vector was digested with EcoRI and ClaI and ligated to a 138 bp PCR fragment amplified from the 0.6 kb EcoRI fragment (base pairs 3789 to 4416 in the Genbank X68203 sequence) which encodes the 3' end of Flt4s shown in FIG. 1 of Pajusola et al., *Cancer Res.* 52:5738-5743, 1992, using the oligonucleotides 5'-CGGAATTCCC CATGACCCCAAC-3' (SEQ ID NO: 4) (forward, EcoRI site underlined) and 5'-CCATCGATGG ATCCTACCTG AAGCCGCTTT CTT-3' (SEQ ID NO: 5) (reverse, ClaI site underlined). The coding domain was completed by ligation of the 1.2 kb EcoRI fragment (base pairs 2535-3789 of sequence X68203) into the above construct. The complete cDNA was subcloned as a HindIII-ClaI (blunted) fragment (this ClaI site was also included in the 3' primer used to construct the 3' end of the coding sequence) to the pLTRpoly expression vector reported in Mäkelä et al., *Gene*, 118: 293-294 (1992) (Genbank accession number X60280), incorporated by reference herein, using its HindIII-Acc I (blunted) restriction sites:

The long form of Flt4 was produced by replacing the 3'-end of the short form as follows: The 3' region of the Flt4 cDNA was PCR-amplified using a gene specific and a pGEM 3Z vector specific (SP6 promoter) oligonucleotide 5'-ATTAGGTGACACTATA-3' (SEQ ID NO: 6) as reverse and forward primers, respectively, and an Flt4 cDNA clone containing a 495 bp EcoRI fragment extending downstream of the EcoRI site at nucleotide 3789 of the Genbank X68203 sequence (the sequence downstream of this EcoRI site is deposited as the Flt4 long form 3' sequence having Genbank accession number S66407). The gene specific oligonucleotide contained a BamHI restriction site located right after the end of the coding region. The sequence of that (reverse primer) oligonucleotide was 5'-CCATCGATGCATCCCGATGCTGCTTAGTAGCTGT-3' (SEQ ID NO: 7) (BamHI site is underlined). The PCR product was digested with EcoRI and BamHI and transferred in frame to LTRFlt4s vector fragment from which the coding sequences downstream of the EcoRI site at base pair 2535 (see sequence X68203) had been removed by EcoRI-BamHI digestion. Again, the coding domain was completed by ligation of the 1.2 kb EcoRI fragment (base pairs 2535-3789 of sequence X68203) back into the resulting construct.

#### EXAMPLE 2

##### Production and Analysis of Flt4 Transfected Cells

NIH3T3 cells (60% confluent) were co-transfected with 5 µg of the pLTRFlt4 construct and 0.25 µg of the pSV2neo vector (ATCC) containing the neomycin phosphotransferase gene, using the DOTAP liposome-based transfection reagents (Boehringer Mannheim, Mannheim, Germany). One day after the transfection the cells were transferred into selection media containing 0.5 mg/ml geneticin (GIBCO, Grand Island, N.Y.). Colonies of geneticin-resistant cells were isolated and analyzed for expression of the Flt4 proteins. Cells were lysed in boiling lysis buffer containing 3.3% SDS (sodium dodecyl sulphate), 125 mM Tris, pH 6.8. Protein concentrations of the samples were measured by the BCA method (Pierce, Rockford, Ill.). About 50 µg of protein of each lysate were analyzed for the presence of Flt4 by 6% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting using antisera against the carboxyl terminus of Flt4 and the ECL method (Amersham).

For production of anti-Flt4 antiserum the Flt4 cDNA fragment encoding the 40 carboxy-terminal amino acid residues of the short form: NH<sub>2</sub>-PMTPTTYKG SVDN-

QTDSGM VLASEEFQI ESRHQESGFR-COOH (SEQ ID NO: 8) was cloned as a 657 bp EcoRI-fragment into the pGEX-1λT bacterial expression vector (Pharmacia) in frame with the glutathione-S-transferase coding region. The resultant GST-Flt4S fusion protein was produced in *E. coli* and purified by affinity chromatography using a glutathione-Sepharose 4B column. The purified protein was lyophilized, dissolved in phosphate buffered saline (PBS), mixed with Freund's adjuvant and used for immunization of rabbits at biweekly intervals using methods standard in the art (Harlow and Lane, *Antibodies*, A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1988). Antisera were used after the fourth booster immunization for immunoprecipitation of Flt4 from the transfected cells and cell clones expressing Flt4 were used for ligand stimulation analysis.

#### EXAMPLE 3

##### Construction of a Flt4 EC Baculovirus Vector and Expression and Purification of its Product

The construction of an Flt4 extracellular domain (EC) baculovirus vector is schematically shown in FIG. 3. The Flt4-encoding cDNA has been prepared in both a long form and a short form, each being incorporated in a vector under control of the Moloney murine leukemia virus LTR promoter. The nucleotide sequence of the short form of the Flt4 receptor is available on the Genbank database as Accession No. X68203 and the specific 3' segment of the long form cDNA is available as Accession No. S66407.

The ends of a cDNA segment encoding Flt4 extracellular domain (EC) were modified as follows: The 3' end of Flt4 cDNA sequence (Genbank Accession Number X68203) which encodes the extracellular domain was amplified using primer 1116 5'-CTGGAGTCCGACTTGGCGGACT-3' (SEQ ID NO: 9, SalI site underlined) and primer 1315 5'-CGCGGATCCCTAGTGATGGTGATGGTGATGTCTACCTTCGATCATGCT GCCCTTAT CCTC-3' (SEQ ID NO: 10, BamHI site underlined). The sequence complementary to that of primer 1315 continues after the Flt4 reading frame and encodes 6 histidine residues for binding to a Ni-NTA column (Qiagen, Hilden, Germany) followed by a stop codon, and an added Bam HI site. The amplified fragment was digested with SalI and BamHI and used to replace a unique SalI-BamHI fragment in the LTRFlt4 vector shown in FIG. 3. The SalI-BamHI fragment that was replaced encodes the Flt4 transmembrane and cytoplasmic domains.

The 5' end without the Flt4 signal sequence encoding region was amplified by PCR using the primer 1335 5'-CCCAAGCTTGGATCCAAGTGGCTACTCCATGACC-3' (SEQ ID NO: 11) (the primer contains added HindIII (AAGCTT) and BamHI (GGATCC) restriction sites, which are underlined) and primer 1332 5'-GTTGCCTGTGATGTGCACCA-3' (SEQ ID NO: 12). The amplified fragment was digested with HindIII and SphI (the HindIII site (AAGCTT) is underlined in primer 1335 and the SphI site is within the amplified region of the Flt4 cDNA). The resultant HindIII-SphI fragment was used to replace a HindIII-SphI fragment in the modified LTRFlt4 vector described immediately above (the HindIII site is in the 5' junction of the Flt4 insert with the pLTRpoly portion of the vector, the SphI site is in Flt4 cDNA). The resultant Flt4EC insert was then ligated as a BamHI fragment into the BamHI site in the pVTBac plasmid as disclosed in Tessier et al., *Gene* 98: 177-183 (1991), incorporated by reference herein. The orientation was confirmed to be correct by partial sequencing so that the open reading frame of the signal sequence-encoding portion of the vector continued in

frame with the Flt4 sequence. That construct was transfected together with the baculovirus genomic DNA into SF-9 cells by lipofection. Recombinant virus was purified, amplified and used for infection of High-Five cells (Invitrogen, San Diego, Calif.) using methods standard in the art. The Flt4 extracellular domain (Flt4EC) was purified from the culture medium of the infected High-Five cells using Ni-NTA affinity chromatography according to manufacturer's instructions (Qiagen) for binding and elution of the 6xHis tag encoded in the COOH-terminus of the recombinant Flt4 extracellular domain.

#### EXAMPLE 4

##### Isolation of Flt4 Ligand from Conditioned Media

An Flt4 ligand according to the invention was isolated from conditioned media from PC-3 prostatic adenocarcinoma cell line CRL1435 from the American Type Culture Collection and cultured as instructed by the supplier in Ham's F-12 Nutrient mixture (GIBCO) containing 7% fetal calf serum. In order to prepare the conditioned media, confluent PC-3 cells were cultured for 7 days in Ham's F-12 Nutrient mixture (GIBCO) in the absence of fetal bovine serum. Medium was then cleared by centrifugation at 10,000 g for 20 minutes. The medium was then screened to determine its ability to induce tyrosine phosphorylation of Flt4 by exposure to NIH3T3 cells which had been transfected with Flt4-encoding cDNA using the pLTRFlt4 vector. For receptor stimulation experiments, subconfluent NIH3T3 cells were starved overnight in serum-free DMEM medium (GIBCO) containing 0.2% BSA. The cells were stimulated with the conditioned media for 5 minutes, washed twice with cold PBS containing 100  $\mu$ M vanadate and lysed in RIPA buffer (10 mM Tris pH 7.5, 50 mM NaCl, 0.5% sodium deoxycholate, 0.5% Nonidet P40 (BDH, Poole, England), 0.1% SDS, 0.1 U/ml Aprotinin (Boehringer Mannheim), 1 mM vanadate) for receptor immunoprecipitation analysis. The lysates were centrifuged for 20 minutes at 15,000 $\times$ g. The supernatants were incubated for 2 hours on ice with 3  $\mu$ l of the antiserum against the Flt4 C-terminus described in Example 2 and also in Pajusola, et al. *Oncogene* 8: 2931-2937, (1993), incorporated by reference herein.

After a 2 hour incubation in the presence of anti-Flt4 antiserum, protein A-Sepharose (Pharmacia) was added and incubation was continued for 45 minutes with rotation. The immunoprecipitates were washed three times with the immunoprecipitation buffer and twice with 10 mM Tris, pH 7.5 before analysis in SDS-PAGE. Polypeptides were transferred to nitrocellulose and analyzed by Western blotting using Flt4- or phosphotyrosine-specific antisera and the ECL method (Amersham International, Buckinghamshire, England). Anti-phosphotyrosine monoclonal antibodies (anti-PTyr; PY20) were purchased from Transduction Laboratories (Lexington, Ky.). In some cases, the filters were restained with a second antibody after stripping. The stripping of the filters was done for 30 minutes at 5 $^{\circ}$  C. in 100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7 with occasional agitation.

As shown in FIG. 4, the PC-3 cell conditioned medium stimulated tyrosine phosphorylation of a 125 kD polypeptide when Flt4-expressing NIH3T3 cells were treated with the indicated preparations of media, lysed, and the lysates were immunoprecipitated with anti-Flt4 antiserum followed by SDS-PAGE, Western blotting, and staining using anti-PTyr antibodies. The resulting band was weakly phosphorylated upon stimulation with unconcentrated PC-3 conditioned medium (lane 2). The 125 kD band comigrated with

the tyrosine phosphorylated, processed form of the mature Flt4 from pervanadate-treated cells (compare lanes 2 and 7 of FIG. 4, see also FIG. 5A). Comigration was confirmed upon restaining with anti-Flt4 antibodies as is also shown in FIG. 5A (panel on the right). In order to show that the 125 kD polypeptide is not a non-specific component of the conditioned medium reactive with anti-phosphotyrosine antibodies, 15  $\mu$ l of conditioned medium were separated by SDS-PAGE, blotted on nitrocellulose and the blot was stained with anti-PTyr antibodies. No signal was obtained (FIG. 5B). Also, unconditioned medium failed to stimulate Flt4 phosphorylation, as shown in FIG. 4, lane 1.

As shown in FIG. 4, lane 3, stimulating activity was considerably increased when the PC-3 conditioned medium was concentrated four-fold using a Centricon-10 concentrator (Amicon). FIG. 4, lane 4, shows that pretreatment of the concentrated PC-3 conditioned medium with 50  $\mu$ l of the Flt4 extracellular domain coupled to CNBr-activated sepharose CL-4B (Pharmacia; about 1 mg of Flt4EC domain/ml sepharose resin) completely abolished Flt4 tyrosine phosphorylation. Similar pretreatment of the conditioned medium with unsubstituted sepharose CL-4B did not affect stimulatory activity, as shown in FIG. 4, lane 5. Also, the flow through obtained after concentration, which contained proteins of less than 10,000 molecular weight, did not stimulate Flt4 phosphorylation, as shown in FIG. 4, lane 6.

The foregoing data show that PC-3 cells produce a ligand which binds to the extracellular domain of Flt4 and activates this receptor.

#### EXAMPLE 5

##### Purification of the Flt4 Ligand

The ligand expressed by PC-3 cells as characterized in Example 4 was purified and isolated using a recombinantly-produced Flt4 extracellular domain (Flt4EC) in affinity chromatography.

Two harvests of serum-free conditioned medium, comprising a total of 8 L, were collected from 500 confluent 15 cm diameter culture dishes containing confluent layers of PC-3 cells. The conditioned medium was clarified by centrifugation at 10,000 $\times$ g and concentrated 80-fold using an Ultrasette Tangential Flow Device (Filtron, Northborough, Mass.) with a 10 kD cutoff Omega Ultrafiltration membrane according to the manufacturer's instructions. Recombinant Flt4 extracellular domain was expressed in a recombinant baculovirus cell system and purified by affinity chromatography on Ni-agarose (Ni-NTA affinity column obtained from Qiagen). The purified extracellular domain was coupled to CNBr-activated Sepharose CL-4B at a concentration of 5 mg/ml and used as an affinity matrix for ligand affinity chromatography.

Concentrated conditioned medium was incubated with 2 ml of the recombinant Flt4 extracellular domain-Sepharose affinity matrix in a rolling tube at room temperature for 3 hours. All subsequent purification steps were at 4 $^{\circ}$  C. The affinity matrix was then transferred to a column (Pharmacia) with an inner diameter of 15 mm and washed successively with 100 ml of PBS and 50 ml of 10 mM Na-phosphate buffer (pH 6.8). Bound material was eluted step-wise with 100 mM glycine-HCl, successive 6 ml elutions having pHs of 4.0, 2.4, and 1.9. Several 2 ml fractions of the eluate were collected in tubes containing 0.5 ml 1 M Na-phosphate (pH 8.0). Fractions were mixed immediately and dialyzed in 1 mM Tris-HCl (pH 7.5). Aliquots of 75  $\mu$ l each were analyzed

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for their ability to stimulate tyrosine phosphorylation of Flt4. The ultrafiltrate, 100  $\mu$ l aliquots of the concentrated conditioned medium before and after ligand affinity chromatography, as well as 15-fold concentrated fractions of material released from the Flt4 extracellular domain-Sephacrose matrix during the washings were also analyzed for their ability to stimulate Flt4 tyrosine phosphorylation.

As shown in FIG. 6, lane 3, the concentrated conditioned medium induced prominent tyrosine phosphorylation of Flt4 in transfected NIH3T3 cells overexpressing Flt4. This activity was not observed in conditioned medium taken after medium was exposed to the Flt4 Sepharose affinity matrix described above (lane 4). The specifically-bound Flt4-stimulating material was retained on the affinity matrix upon washes in PBS, 10 mM Na-phosphate buffer (pH 6.8), and at pH 4.0 (lanes 5-7, respectively), and it was eluted in the first two 2 ml aliquots at pH 2.4 (lanes 8 and 9). A further decrease of the pH of the elution buffer did not cause release of additional Flt4-stimulating material (lane 11).

Small aliquots of the chromatographic fractions were concentrated in a SpeedVac concentrator (Savant, Farmingdale, N.Y.) and subjected to SDS-PAGE under reducing conditions with subsequent silver staining of the gel. As shown in FIG. 7, the major polypeptide, having a molecular weight of approximately 23 kD (reducing conditions), was detected in the fractions containing Flt4 stimulating activity (corresponding to lanes 8 and 9 in FIG. 6). That polypeptide was not found in the other chromatographic fractions. On the other hand, all other components detected in the two active fractions were also distributed in the starting material and in small amounts in the other washing and elution steps after their concentration. Similar results were obtained in three independent affinity purifications, indicating that the 23 kD polypeptide specifically binds to Flt4 and induces its tyrosine phosphorylation.

Fractions containing the 23 kD polypeptide were combined, dried in a SpeedVac concentrator and subjected to SDS-PAGE in a 12.5% gel. The proteins from the gel were then electroblotted to Immobilon-P (PVDF) transfer membrane (Millipore, Marlborough, Mass.) and visualized by staining of the blot with Coomassie blue R-250. The region containing only the stained 23 kD band was cut from the blot and was subjected to N-terminal amino acid sequence analysis in a Prosite Protein Sequencing System (Applied Biosystems, Foster City, Calif.). The data were analyzed using a 610A Data Analysis System (Applied Biosystems). Analysis revealed a single N-terminal sequence of NH<sub>2</sub>-XEETIKFAAAHYNTEILK-COOH (SEQ ID NO: 13).

#### EXAMPLE 6

##### Construction of PC-3 Cell cDNA Library in a Eukaryotic Expression Vector

Poly(A)<sup>+</sup> RNA was isolated from five 15 cm diameter confluent dishes of PC-3 cells by a single step method using oligo(dT) (Type III, Collaborative Research) cellulose affinity chromatography (Sambrook et al., Molecular Cloning, A Laboratory Manual; Cold Spring Harbor Laboratory Press, 1989). The yield was 70  $\mu$ g. Six micrograms of the Poly(A)<sup>+</sup> RNA were used to prepare an oligo(dT)-primed cDNA library in the mammalian expression vector pcDNA I and the Librarian kit of Invitrogen according to the instructions included in the kit. The library was estimated to contain about 10<sup>6</sup> independent recombinants with an average insert size of approximately 1.8 kb.

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#### EXAMPLE 7

##### Amplification of the Unique Nucleotide Sequence Encoding the Flt4 Ligand

Degenerate oligonucleotides were designed based on the N-terminal amino acid sequence of the isolated Flt4 ligand and were used as primers in a polymerase chain reaction (PCR) to amplify cDNA encoding the Flt4 ligand from a PC-3 cell library. The overall strategy is schematically depicted in FIG. 9A, where the different primers have been marked with arrows.

The PCR was carried out using 1  $\mu$ g of DNA from the amplified PC-3 cDNA library and a mixture of sense-strand primers comprising 5'-GCAGARGARACNATHAA-3' (SEQ ID NO: 14) (wherein R is A or G, N is A, G, C or T and H is A, C or T), encoding amino acid residues 2-6 (EETIK, SEQ ID NO: 15) and antisense-strand primers 5'-GCAYTTNARDATYTCNGT-3' (SEQ ID NO: 16) (wherein Y is C or T and D is A, G or T), corresponding to amino acid residues 14-18 (TEILK, SEQ ID NO: 17). Three extra nucleotides (GCA) were added to the 5'-terminus of each primer to increase annealing stability. Two successive PCR runs were carried out using 1 U per reaction of DynaZyme (F-500L, Finnzymes), a thermostable DNA polymerase, in a buffer supplied by the manufacturer (10 mM Tris-HCl, pH 8.8 at 25° C., 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.1% Triton-X100), at an extension temperature of 72° C. The first PCR run was carried out for 43 cycles. The first three cycles were run at an annealing temperature of 33° C. for 2 minutes, and the remaining cycles were run at 42° C. for 1 minute.

The region of the gel containing a weak band of the expected size (57 bp) was cut out from the gel and eluted. The eluted material was reamplified for 30 cycles using the same primer pairs described above at 42° C. for 1 minute. The amplified fragment was cloned into a pCR II vector (Invitrogen) using the TA cloning kit (Invitrogen) and sequenced using the radioactive dideoxynucleotide sequencing method of Sanger. Six clones were analyzed and all contained the sequence encoding the expected peptide (amino acids 2-18 of the Flt4 ligand precursor). Nucleotide sequence spanning the region from the third nucleotide of codon 6 to the third nucleotide of codon 13 (the extension region) was identical in all six clones: 5'-ATTGCTGCAGCACACTACAAC-3' (SEQ ID NO: 18) and thus was considered to represent an amplified product from the unique sequence encoding part of the amino terminus of the Flt4 ligand.

#### EXAMPLE 8

##### Amplification of the 5'-end of the cDNA Encoding the Flt4 Ligand

Based on the unique nucleotide sequence encoding the N-terminus of the isolated Flt4 ligand, two pairs of nested primers were designed to amplify, in two subsequent PCR-reactions, the complete 5'-end of the corresponding cDNAs from one microgram of DNA from the above-described PC-3 cDNA library. First, amplification was performed with primer 5'-TCNGTGTTGTAGTGTGCTG-3' (SEQ ID NO: 19), which is the antisense-strand primer corresponding to amino acid residues 9-15 (AAHYNTE, SEQ ID NO: 20), and sense-strand primer 5'-TAATACGACTCACTATAGGG-3' (SEQ ID NO: 21), corresponding to the T7 RNA promoter of the pcDNAI vector used for construction of the library. "Touchdown"



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PCR was used as disclosed in Don, et al., *Nucl. Acids Res.*, 19: 4008 (1991), incorporated by reference herein. The annealing temperature of the two first cycles was 62° C. and subsequently the annealing temperature was decreased in every other cycle by 1° C. until a final temperature of 53° C. was reached, at which temperature 16 additional cycles were conducted. Annealing time was 1 minute and extension at each cycle was conducted at 72° C. for 1 minute. Multiple amplified DNA fragments were obtained in the first reaction. The products of the first amplification (1 ul of a 1:100 dilution in water) were used in the second amplification reaction employing the nested primers 5'-GTTGTAGTGTGCTGCAGCGAATT-3' (SEQ ID NO: 22); an antisense-strand primer corresponding to amino acid residues 6-13 (KFAAAHYN, SEQ ID NO: 23) of the Flt4 ligand, and 5'-TCACTATAGGGAGACCCAAGC-3' (SEQ ID NO: 24), a sense-strand primer corresponding to nucleotides 2179-2199 of the pcDNAI vector. The sequences of these sense and antisense primers overlapped with the 3' ends of the corresponding primers used in the first PCR. "Touchdown" PCR was carried out by decreasing the annealing temperature from 72° C. to 66° C. and continuing with 18 additional cycles at 66° C. The annealing time was 1 minute and extension at each cycle was carried out at 72° C. for 2 minutes. One major product of about 220 bp and three minor products of about 270 bp, 150 bp, and 100 bp were obtained.

The amplified fragment of approximately 220 bp was cut out from the agarose gel, cloned into a pCRII vector using the TA cloning kit (Invitrogen) and sequenced. Three recombinant clones were analyzed and they contained the sequence

5'-TCACTATAGGGAGACCCAAGCTTGGTACCGAGCT  
CGGATCCACTAGT AACGGCCGCCAGTGTGGTG-  
GAATTCGACGAATCATGACTGTACTCT  
ACCCAGAATATTGAAAATGTACAAGTGTACGCTAA  
GGCAAGGAGGC  
TGGCAACATAACAGAGAACAGGCCAACCTCAACTC  
AAGGACAGAAG

AGACTATAAAATTTCGCTGCAGCACACTACAAC-3' (SEQ ID NO: 25). The beginning of the sequence represents the pcDNAI vector and the underlined sequence represents the amplified product of the 5'-end of the insert. The ATG codon located upstream of that sequence in the same reading frame is followed by an open reading frame containing the amplified product of the putative signal sequence and the first 13 amino acid residues of the secreted Flt4 ligand. The cloning of the 5' end of the Flt4 cDNA, as described in the preceding two examples, is depicted schematically in FIG. 9A.

#### EXAMPLE 9

##### Amplification of the 3'-end of cDNA Encoding the Flt4 Ligand

Based upon the amplified 5'-sequence of the clones encoding the Flt4 ligand, two pairs of non-overlapping nested primers were designed to amplify the 3'-portion of the FLT4-L clones. The sense-strand primer 5'-ACAGAGAACAGGCCAACCC-3' (SEQ ID NO: 26) and antisense-strand primer 5'-TCTAGCAATTAGGTGACAC-3' (SEQ ID NO: 27) corresponding to nucleotides 2311-2329 of the pcDNAI vector were used in a first "touchdown" PCR. The annealing temperature of the reaction was decreased 1° C. every two cycles from 72° C. to 52° C., at which temperature 15 additional cycles were carried out. The annealing time was 1 minute and extension at each cycle was carried out at 72° C. for 3 minutes. DNA fragments of several sizes were obtained in the first amplifica-

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tion. Those products were diluted 1:200 in water and reamplified in PCR using the second pair of primers: 5'-AAGAGACTATAAAATTTCGCTGCAGC-3' (SEQ ID NO: 28) and 5'-CCCTCTAGATGCATGCTCGA-3' (SEQ ID NO: 29) (antisense-strand primer corresponding to nucleotides 2279-2298 of the pcDNAI vector). Two DNA fragments were obtained, having sizes of 1350 bp and 570 bp. Those fragments were cloned into a pCRII vector and the inserts of the clones were sequenced. Both of these fragments were found to contain sequences encoding an amino acid sequence homologous to the VEGF sequence.

#### EXAMPLE 10

##### Screening the PC-3 Cell cDNA Library Using the 5' PCR Fragment of Flt4 Ligand cDNA

A 219 bp 5'-terminal fragment of Flt4 ligand cDNA was amplified by PCR using the 5' PCR fragment described above and primers 5'-GTTGTAGTGTGCTGCAGCGAATT-3' (antisense-strand primer, SEQ ID NO: 30) and 5'-TCACTATAGGGAGACCCAAGC-3' (SEQ ID NO: 31) (sense-primer corresponding to nucleotides 2179-2199 of the pcDNAI vector). The amplified product was subjected to digestion with EcoRI (Boehringer Mannheim) to remove the portion of the DNA sequence amplified from the pcDNAI vector and the resulting 153 bp fragment encoding the 5' end of the Flt4 ligand was labeled with [<sup>32</sup>P]-dCTP using the Klenow fragment of *E. coli* DNA polymerase I (Boehringer Mannheim). That fragment was used as a probe for hybridization screening of the amplified PC-3 cell cDNA library.

Filter replicas of the library were hybridized with the radioactively labeled probe at 42° C. for 20 hours in a solution containing 50% formamide, 5xSSPE, 5xDenhardt's solution, 0.1% SDS and 0.1 mg/ml denatured salmon sperm DNA. Filters were washed twice in 1xSSC, 0.1% SDS for 30 minutes at room temperature, then twice for 30 minutes at 65° C. and exposed overnight.

On the basis of autoradiography, 10 positive recombinant bacterial colonies hybridizing with the probe were chosen from the library. Plasmid DNA was purified from these colonies and analyzed by EcoRI and NotI digestion and agarose gel electrophoresis followed by ethidium bromide staining. The ten plasmid clones were divided into three groups on the basis of the presence of insert sizes of approximately 1.7, 1.9 and 2.1 kb, respectively. Inserts of plasmids from each group were sequenced using the T7 oligonucleotide as a primer and walking primers for subsequent sequencing reactions.

Sequence analysis showed that all clones contain the open reading frame encoding the NH2-terminal sequence of the Flt4 ligand. Furthermore, the 2.1 and 1.9 kb clones also contained sequences encoding the signal sequence (FIG. 9A, SS). The 5' end of the 1.7 kb clone began within the signal sequence-encoding portion. Dideoxy sequencing was continued using walking primers in the downstream direction. An 1140 nucleotide portion of the sequence of the longest clone is shown in FIGS. 9B through 9D. As can be seen in that figure, after the putative signal sequence the open reading frame terminates in a TAA stop codon 318 amino acid residues further downstream from the 33 amino acid signal sequence. When compared with sequences in the GenBank Database, the predicted protein product of this reading frame was found to be homologous with the predicted amino acid sequences of the PDGF/VEGF family of growth factors, as shown in FIGS. 10A through 10D.

## EXAMPLE 11

## Stimulation of Flt4 Autophosphorylation by the Protein Product of the Flt4 Ligand Vector

The 2.1 kb insert of the Flt4-L clone in pcDNA1 vector containing the open reading frame encoding the sequence shown in FIGS. 9B through 9D (SEQ ID NO: 32) was cut out from the vector using HindIII and NotI restriction enzymes, isolated from a preparative agarose gel and ligated to the corresponding sites in the pREP7 expression vector (Invitrogen). The pREP7 vector containing the above cloned insert was transfected into 293-EBNA cells (Invitrogen) using the calcium phosphate transfection method (Sambrook et al., Molecular Cloning, A Laboratory Manual; Cold Spring Harbor Laboratory Press, 1989). About 48 hours after transfection the medium of the transfected cells was changed to DMEM medium lacking fetal calf serum and incubated for 36 h. The thus conditioned medium was then collected, centrifuged at 5000xg for 20 minutes, the supernatant was concentrated 5-fold using Centrprep 10 (Amicon) and used to stimulate NIH3T3 cells expressing LTRFlt4L, as in Example 4. The cells were lysed, immunoprecipitated using anti-Flt4 antiserum and analyzed by Western blotting using anti-phosphotyrosine antibodies.

As can be seen from FIG. 11, lanes 1 and 3, the conditioned medium from two different dishes of the transfected cells stimulated Flt4 autophosphorylation in comparison with the medium from mock-transfected cells, which gave only background levels of phosphorylation of the Flt4 receptor (lane 2). When the concentrated conditioned medium was pre-absorbed with 20  $\mu$ l of a slurry of Flt4EC domain coupled to Sepharose (see example 4), no phosphorylation was obtained (lane 4), showing that the activity responsible for Flt4 autophosphorylation was indeed the Flt4 ligand. Thus, these results demonstrate that the Flt4-L plasmid vector clone having an approximately 2.1 kb insert and containing the open reading frame shown in FIG. 9B is expressed into a Flt4 ligand in cells transfected with the Flt4-L expression vector clone, and thus is biologically active. The sequence encoded by that open reading frame is shown in SEQ ID NO: 33. Plasmid pFLT4-L has been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md. 20852 as accession number 97231. The approximately 2.1 kb cDNA insert of the deposited plasmid pFLT4-L was sequenced and found to have a nucleotide sequence that includes the 1997 nucleotides of sequence set forth in SEQ ID NO: 44. The nucleotide sequence set forth in SEQ ID NO: 44 encodes the 419 residue amino acid sequence set forth in SEQ ID NO: 45.

However, the predicted molecular weight of the mature protein product deduced from the reading frame specified in SEQ ID NOs: 32-33 is 35881 and the Flt4 ligand from PC-3 cell cultures had an approximate molecular weight of 23 kD under reducing conditions. It is thus possible that the Flt4-L mRNA may be first translated into a precursor, from which the mature ligand is derived by proteolytic cleavage. The difference in the observed molecular weight of the isolated Flt4 ligand and the deduced molecular weight of the disclosed open reading frame of the Flt4 ligand sequence may then derive from sequences in the carboxyl terminal region of the latter. Also, the Flt4 ligand may be glycosylated at two putative N-linked glycosylation sites conforming to the consensus which can be identified in the deduced Flt4 ligand amino acid sequence (N-residues underlined in FIGS. 10B and 10C).

The carboxyl terminal amino acid sequences, which increase the predicted molecular weight of the Flt4 ligand

subunit in comparison with other ligands of this family, show a pattern of spacing of cysteine residues reminiscent of the Balbiani ring protein 3 (BR3P) sequence (Dignam and Case, Gene 88, 133-140, 1990), as depicted in FIG. 9A. Such a sequence may encode an independently folded domain present in a Flt4 ligand precursor and it may be involved, for example, in the regulation of secretion, solubility, stability, cell surface localization or activity of the Flt4 ligand. Interestingly, at least one cysteine motif of the BR3P type is also found in the VEGF carboxy terminal amino acid sequences.

Thus, the Flt4-L mRNA may be first translated into a precursor from the mRNA corresponding to the Flt4-L clone, from which the mature ligand is derived by proteolytic cleavage. To define the mature Flt4 ligand product one first expresses the cDNA clone, which is deposited in the pcDNA1 expression vector, in cells, such as COS cells. One uses antibodies generated against Flt4-L-encoded peptides, such as amino terminal 23 amino acid peptide or bacterial Flt4 fusion proteins; such as a GST-fusion protein, to raise antibodies against the VEGF-homologous domain of Flt4 ligand. One then follows the biosynthesis and processing of the Flt4 ligand in the transfected cells by pulse-chase analysis using radioactive cysteine for labelling of the cells, immunoprecipitation and gel electrophoresis. Using antibodies against the two domains of the product of the Flt4-L clone material for radioactive or nonradioactive aminoterminal sequence analysis is isolated. The determination of the NH<sub>2</sub>-terminal sequence of the carboxyl terminal fragment allows for identification of the proteolytic processing site. This is confirmed by site-directed mutagenesis of the amino acid residues adjacent to the cleavage site, which would prevent the cleavage.

On the other hand, the Flt4 ligand is characterized by progressive 3' deletions in the 3' coding sequences of the Flt4 ligand precursor clone, resulting in carboxy-terminal truncations of its protein product. The activities of such truncated forms are assayed by, for example, studying Flt4 autophosphorylation induced by the truncated proteins when applied to cultures of cells, such as NIH3T3 cells expressing LTRFlt4. By extrapolation from studies of the structure of the related platelet derived growth factor (PDGF, reference Heldin et al., *Growth Factors* 8:245-252 (1993)) one determines that the region critical for receptor activation by the Flt4 ligand is contained within its first approximately 180 amino acid residues of the secreted VEGF-C protein lacking the signal sequence, and apparently within the first approximately 120 amino acid residues.

On the other hand, the difference between the molecular weights of the purified ligand and the open reading frame of the Flt4 precursor clone may be due to the fact that the soluble ligand was produced from an alternatively spliced mRNA which would also be present in the PC-3 cells, from which the isolated ligand was derived. To isolate such alternative cDNA clones one uses cDNA fragments of the deposited clone and PCR primers made according to the sequence provided as well as techniques standard in the art to isolate or amplify alternative cDNAs from the PC-3 cell cDNA library. One may also amplify using reverse transcription (RT)-PCR directly from the PC-3 mRNA using the primers provided in the sequence of the Flt4-L clone. Alternative cDNA sequences are determined from the resulting cDNA clones. One can also isolate genomic clones corresponding to the Flt4-L transcript from a human genomic DNA library using methods standard in the art and to sequence such clones or their subcloned fragments to reveal the corresponding exons. Alternative exons can then

be identified by a number of methods standard in the art, such as heteroduplex analysis of cDNA and genomic DNA, which are subsequently characterized.

#### EXAMPLE 12

##### Expression of the Flt4L Gene

Expression of transcripts corresponding to the Flt4 ligand (VEGF-C) was analyzed by hybridization of Northern blots containing isolated poly(A)<sup>+</sup> RNA from HT-1080 and PC-3 human tumor cell lines. The probe was the radioactively labelled insert of the 2.1 kb cDNA clone (specific activity 10<sup>8</sup>–10<sup>9</sup> cpm/mg of DNA). The blot was hybridized overnight at 42° C. using 50% formamide, 5×SSPE buffer, 2% SDS, 10×Denhardt's solution, 100 mg/ml salmon sperm DNA and 1×10<sup>6</sup> cpm of the labelled probe/ml. The blot was washed at room temperature for 2×30 minutes in 2×SSC containing 0.05% SDS, and then for 2×20 min at 52° C. in 0.1×SSC containing 0.1% SDS. The blot was then exposed at -70° C. for three days using intensifying screens and Kodak XAR film. Both cell lines expressed an Flt4 ligand mRNA of about 2.3 kb, as well as VEGF and VEGF-B mRNAs (FIG. 12).

#### EXAMPLE 13

##### VEGF-C Chains are Proteolytically Processed after Biosynthesis and Disulfide Linked

The predicted molecular mass of the secreted polypeptide, as deduced from the VEGF-C ORF specified in SEQ ID NOs: 32 and 33, is 35,881 kD, suggesting that VEGF-C mRNA may be first translated into a precursor, from which the mature ligand of 23 kD is derived by proteolytic cleavage.

To study this, metabolic labelling of 293 EBNA cells transfected with the VEGF-C construct was carried out by addition of 100  $\mu$ Ci/ml of Pro-mix<sup>TM</sup> L-[<sup>35</sup>S] in vitro cell labelling mix (Amersham) to the culture medium devoid of cysteine and methionine. After two hours, the cell layers were washed twice with PBS and the medium was then replaced with DMEM-0.2% BSA. After 1, 3, 6, 12 and 24 hours of subsequent incubation, the culture medium was collected, clarified by centrifugation, and concentrated, and VEGF-C was bound to 30  $\mu$ l of a slurry of Flt4EC-Sepharose overnight at +4° C., followed by three washes in PBS, two washes in 20 mM Tris-HCl (pH 7.5), alkylation, SDS-PAGE and autoradiography.

These experiments demonstrated that a putative precursor polypeptide of 32 kD apparent molecular mass was bound to the Flt4EC affinity matrix from the CM of metabolically labelled cells transfected with a VEGF-C expression vector (FIG. 13A). Increased amounts of a 23 kD receptor-binding polypeptide accumulated in the culture medium during a subsequent chase period of 3 h, but not thereafter (lanes 2–4 and data not shown), suggesting that the 23 kD form is produced by proteolytic processing, which is cell-associated and incomplete, at least in the transiently transfected cells. The arrows in FIG. 13A indicate the 32 kDa and 23 kDa polypeptides of secreted VEGF-C.

In a related experiment, VEGF-C isolated using Flt4EC-Sepharose after a 4 h continuous metabolic labelling was analyzed by polyacrylamide gel electrophoresis in nonreducing conditions (FIG. 13B). Higher molecular mass forms were observed under nonreducing conditions, suggesting that the VEGF-C polypeptides can form disulfide-linked dimers and/or multimers (arrows in FIG. 13B).

#### EXAMPLE 14

##### Stimulation of VEGFR-2 Autophosphorylation by VEGF-C

Conditioned medium (CM) from 293 EBNA cells transfected with the VEGF-C vector also was used to stimulate porcine aortic endothelial (PAE) cells expressing VEGFR-2. Pajusola et al., *Oncogene*, 9:3545–55 (1994); Waltenberger et al., *J. Biol. Chem.*, 269:26988–95 (1994). The cells were lysed and immunoprecipitated using VEGFR-2-specific antiserum (Waltenberger et al., 1994).

PAE-KDR cells (Waltenberger et al., 1994) were grown in Ham's F12 medium-10% fetal calf serum (FCS). Confluent NIH3T3-Flt4 cells or PAE-KDR cells were starved overnight in DMEM or Ham's F12 media, respectively, supplemented with 0.2% bovine serum albumin (BSA) and then incubated for 5 min. with the analyzed media. Recombinant human VEGF (R&D Systems) and PDGF-BB were used as a control stimulating agents. The cells were washed twice with ice-cold tris-buffered saline (TBS) containing 100 mM sodium orthovanadate and lysed in RIPA buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 U/ml aprotinin and 1 mM sodium orthovanadate. The lysates were sonicated, clarified by centrifugation at 16,000 g for 20 min. and incubated for 3–6 h on ice with 3–5  $\mu$ l of antisera specific for Flt4 (Pajusola et al., 1993), VEGFR-2 or PDGFR- $\beta$  (Claesson-Welsh et al., *J. Biol. Chem.*, 264:1742–47 (1989); Waltenberger et al., 1994). Immunoprecipitates were bound to protein A-Sepharose, washed three times with RIPA containing 1 mM PMSF, 1 mM sodium orthovanadate, twice with 10 mM Tris-HCl (pH 7.4) and subjected to SDS-PAGE in a 7% gel. Polypeptides were transferred to nitrocellulose by Western blotting and analyzed using PY20 phosphotyrosine-specific monoclonal antibodies (Transduction Laboratories) or receptor-specific antiserum and ECL method (Amersham).

The results of the experiment are presented in FIGS. 14A and 14B. As shown in FIG. 14A, PAE cells expressing VEGFR-2 were stimulated with 10- or 2-fold concentrated medium from mock-transfected 293-EBNA cells (lanes 1 and 2), or with 2-, 5- or 10-fold concentrated medium from 293-EBNA cell cultures expressing the recombinant VEGF-C (lanes 3–6). VEGFR-2 was immunoprecipitated with specific antibodies and analyzed by SDS-PAGE and Western blotting using phosphotyrosine antibodies. For comparison, the stimulations were carried out with non-conditioned medium containing 50 ng/ml of purified recombinant VEGF (lanes 7 and 8). Lanes 6 and 7 show stimulation with VEGF-C- or VEGF- containing media pretreated with Flt4EC. As depicted in FIG. 14B, PDGFR- $\beta$ -expressing NIH3T3 cells were stimulated with non-conditioned medium (lane 1), 5-fold concentrated CM from mock-transfected (lane 2) or VEGF-C-transfected (lanes 3 and 4) cells, or with non-conditioned medium containing 50 ng/ml of recombinant human PDGF-BB (lane 5). Medium containing VEGF-C was also pretreated with recombinant Flt4EC (lane 4). PDGFR- $\beta$  was immunoprecipitated with specific antibodies and analyzed by SDS-PAGE and Western blotting using phosphotyrosine antibodies with subsequent stripping and reprobing of the membrane with antibodies specific for PDGFR- $\beta$ .

A basal level of tyrosine phosphorylation of VEGFR-2 was detected in cells stimulated by CM from the mock-transfected cells. A further concentration of this medium resulted in only a slight enhancement of VEGFR-2 phosphorylation (FIG. 14A lanes 1 and 2). CM containing



recombinant VEGF-C stimulated tyrosine autophosphorylation of VEGFR-2 and the intensity of the autophosphorylated polypeptide band was increased upon concentration of the VEGF-C CM (lanes 3-5). Furthermore, the stimulating effect was abolished after pretreatment of the medium with the Flt4EC affinity matrix (compare lanes 1, 5 and 6). The maximal effect of VEGF-C in this assay was comparable to the effect of recombinant VEGF added to unconditioned medium at concentration of 50 ng/ml (lane 8). Pretreatment of the medium containing VEGF with Flt4EC did not abolish its stimulating effect on VEGFR-2 (compare lanes 7 and 8). These results suggest that the VEGF-C expression vector encodes a ligand not only for Flt4 (VEGFR-3), but also for VEGFR-2.

In order to further confirm that the stimulating effect of VEGF-C on tyrosine phosphorylation of VEGFR-3 and VEGFR-2 was receptor-specific, we analyzed the effect of VEGF-C on tyrosine phosphorylation of PDGF receptor  $\beta$  (PDGFR- $\beta$ ) which is abundantly expressed on fibroblastic cells. As can be seen from FIG. 14B, a weak tyrosine phosphorylation of PDGFR- $\beta$  was detected upon stimulation of Flt4-expressing NIH3T3 cells with CM from the mock-transfected cells (compare lanes 1 and 2). A similar low level of PDGFR- $\beta$  phosphorylation was observed when the cells were incubated with CM from the VEGF-C transfected cells, with or without prior treatment with Flt4EC (lanes 3 and 4). In contrast, the addition of 50 ng/ml of PDGF-BB induced a prominent tyrosine autophosphorylation of PDGFR- $\beta$  (lane 5).

#### EXAMPLE 15

##### VEGF-C Stimulates Endothelial Cell Migration in Collagen Gel

CM from cell cultures transfected with the VEGF-C expression vector was placed in a well made in collagen gel and used to stimulate the migration of bovine capillary endothelial (BCE) cells in the three-dimensional collagen gel as follows.

BCE cells (Folkman et al., *Proc. Nat'l Acad. Sci. USA*, 76:5217-5221 (1979) were cultured as described in (Pertovaara et al., *J. Biol. Chem.*, 269:6271-74 (1994)). The collagen gels were prepared by mixing type I collagen stock solution (5 mg/ml in 1 mM HCl) with an equal volume of 2xMEM and 2 volumes of MEM containing 10% newborn calf serum to give a final collagen concentration of 1.25 mg/ml. The tissue culture plates (5 cm diameter) were coated with about 1 mm thick layer of the solution, which was allowed to polymerize at 37° C. BCE cells were seeded on top of this layer. For the migration assays, the cells were allowed to attach inside a plastic ring (1 cm diameter) placed on top of the first collagen layer. After 30 min., the ring was removed and unattached cells were rinsed away. A second layer of collagen and a layer of growth medium (5% newborn calf serum (NCS)), solidified by 0.75% low melting point agar (FMC BioProducts, Rockland, Me.), were added. A well (3 mm diameter) was punched through all the layers on both sides of the cell spot at a distance of 4 mm, and the sample or control media were pipetted daily into the wells. Photomicrographs of the cells migrating out from the spot edge were taken after six days through an Olympus CK 2 inverted microscope equipped with phase-contrast optics. The migrating cells were counted after nuclear staining with the fluorescent dye bisbenzimidazole (1 mg/ml, Hoechst 33258, Sigma).

FIG. 15A depicts a comparison of the number of cells migrating at different distances from the original area of

attachment towards wells containing media conditioned by the non-transfected (control) or transfected (mock; VEGF-C; VEGF) cells, 6 days after addition of the media. The number of cells migrating out from the original ring of attachment was counted in five adjacent 0.5 mmx0.5 mm squares using a microscope ocular lens grid and 10xmagnification. Cells migrating further than 0.5 mm were counted in a similar way by moving the grid in 0.5 mm steps. The experiments were carried out twice with similar results, and medium values from the one of the experiments are presented with standard error bars. The photographs in FIG. 15B depict phase-contrast microscopy and fluorescent microscopy of the nuclear staining of BCE cells migrating towards the wells containing media conditioned by the mock-transfected cells or by VEGF-C-transfected cells. The areas shown is approximately 1 mmx1.5 mm, and arrows indicate the borders of the original ring of attachment.

After 6 days of treatment, the cultures were stained and cells at different distances outside of the original ring of attachment were counted using fluorescent nuclear staining and detection with a fluorescence microscope equipped with a grid. A comparison of the numbers of migrating cells in successive 0.5 mmx0.5 mm areas is shown in FIG. 15A. As can be seen from the columns, VEGF-C-containing CM stimulated cell migration more than medium conditioned by the non-transfected or mock-transfected cells but less than medium from cells transfected with a VEGF expression vector. An example of typical phase contrast and fluorescent microscopic fields of cultures stimulated with medium from mock-transfected or VEGF-C transfected cells is shown in FIG. 15B. Daily addition of 1 ng of FGF2 into the wells resulted in the migration of approximately twice the number of cells when compared to the stimulation by CM from VEGF-transfected cells.

#### EXAMPLE 16

##### VEGF-C is Expressed in Multiple Tissues

Northern blots containing 2 micrograms of isolated poly (A)<sup>+</sup> RNA from multiple human tissues (blot from Clontech) were probed with radioactively labelled insert of the 2.0 kb VEGF-C cDNA clone. Northern blotting and hybridization analysis showed that the 2.4 kb RNA and smaller amounts of a 2.0 kb mRNA are expressed in multiple human tissues, most prominently in the heart, placenta, muscle, ovary and small intestine (FIG. 16A). Very little VEGF-C RNA was seen in the brain, liver or thymus and peripheral blood leukocytes (pbl) appeared negative. A similar analysis of RNA from human fetal tissues (FIG. 16B) shows that VEGF-C is highly expressed in the kidney and lung and to a lesser degree in the liver, while essentially no expression is detected in the brain. Interestingly, VEGF expression correlates with VEGF-C expression in these tissues, whereas VEGF-B is highly expressed in all tissues analyzed.

#### EXAMPLE 17

##### The VEGF-C Gene Localizes to Chromosome 4q34

A DNA panel of 24 interspecies somatic cell hybrids, which had retained one or two human chromosomes, was used for the chromosomal localization of the VEGF-C gene (Bios Laboratories, Inc., New Haven, Conn.). Primers were designed to amplify an about 250 bp fragment of the VEGF-C gene from somatic cell hybrid DNA. The primers and conditions for polymerase chain reaction (PCR) were

5'-TGAGTGATTGTAGCTGCTGTG-3' (forward) [SEQ ID NO:34] and 5'-TATTGCAGCAACCCACATCT-3' (reverse) [SEQ ID NO:35] for VEGF-C (94° C., 60s/62° C., 45s/72° C., 60s). The PCR products were evaluated by electrophoresis in 1% agarose gels and visualized by ethidium bromide staining in ultraviolet light. [ $\alpha$ -<sup>32</sup>P]-dCTP-labelled cDNA inserts of a plasmid representing the complete VEGF-C coding domain was used as a probe in Southern blotting and hybridization analysis of the somatic cell hybrid DNAs as instructed by the supplier (Bios Laboratories).

The cell lines for fluorescence in situ hybridization (FISH) were obtained from the American Type Culture Collection (Rockville, Md.). Purified DNA from P1 clones 7660 and 7661 (VEGF-C) (Genome Systems, Inc., St. Louis, Mo.) were confirmed positive by Southern blotting of Eco RI-digested DNA followed by hybridization with the VEGF-C cDNA. The P1 clones were then labelled by nick translation either with biotin-11-dUTP, biotin-14-ATP (Sigma Chemical Co., St. Louis, Mo.) or digoxigenin 11-dUTP (Boehringer Mannheim GmbH, Mannheim, Germany) according to standard protocols. PHA-stimulated peripheral blood lymphocyte cultures were treated with 5-bromodeoxyuridine (BrdU) at an early replicating phase to induce G-banding. See Takahashi et al., *Human Genet.*, 86:14-16 (1995); Lemieux et al., *Cytogenet. Cell Genet.*, 59:311-12 (1992). The FISH procedure was carried out in 50% formamide, 10% dextran sulphate in 2xSSC using well-known procedures. See, e.g., Rytönen et al., *Cytogenet. Cell Genet.*, 68:61-63 (1995); Lichter et al., *Proc. Natl. Acad. Sci. USA*, 85:9664-68 (1988). Repetitive sequences were suppressed with 50-fold excess of Cot-1 DNA (BRL, Gaithersburg, Md.) compared with the labeled probe. Specific hybridization signals were detected by incubating the hybridized slides in labelled antidigoxigenin antibodies, followed by counterstaining with 0.1 mmol/L 4,6-diamino-2-phenylindole. Probe detection for two-color experiments was accomplished by incubating the slides in fluorescein isothiocyanate (FITC)-anti-digoxigenin antibodies (Sigma Chemical Co.) and Texas red-avidin (Vector Laboratories, Burlingame, Calif.) or rhodamine-anti-digoxigenin and FITC-avidin.

Multi-color digital image analysis was used for acquisition, display, and quantification of hybridization signals of metaphase chromosomes. The system contains a PXL camera (Photometrics Inc., Tucson, Ariz.) attached to a PowerMac 7100/Av workstation. IPLab software controls the camera operation, image acquisition and Ludl Filter wheel. At least 50 nuclei were scored. Overlapping nuclei and clusters of cells were ignored. A slide containing normal lymphocyte metaphase spreads and interphase nuclei was included in each experiment to control for the efficiency and specificity of the hybridization.

In order to determine the chromosomal localization of the human VEGF-C gene, DNAs from human rodent somatic cell hybrids containing defined sets of human chromosomes were analyzed by Southern blotting and hybridization with the VEGF-C cDNA probe. Among 24 DNA samples on the hybrid panel, representing different human chromosomes, human-specific signals were observed only in hybrids which contained human chromosome 4. The results were confirmed by PCR of somatic cell hybrid DNA using VEGF-C specific primers, where amplified bands were obtained only from DNAs containing human chromosome 4.

A genomic P1 plasmid for VEGF-C was isolated using specific primers and PCR and verified by Southern blotting and hybridization using a VEGF-C specific cDNA probe.

The chromosomal localization of VEGF-C was further studied using metaphase FISH. Using the P1 probe for VEGF-C in FISH a specific hybridization to the 4q34 chromosomal band was detected in 40 out of 44 metaphases (FIG. 17). Double-fluorochrome hybridization using a cosmid probe specific for the aspartylglucosaminidase (AGA) gene showed that VEGF-C is located just proximal to the AGA gene previously mapped to the 4q34-35 chromosomal band.

Biotin labelled VEGF-C P1 and digoxigenin labeled AGA cosmid probes were hybridized simultaneously to metaphase chromosomes. This experiment demonstrated that the AGA gene is more telomerically located than the VEGF-C gene. The foregoing example demonstrates the utility of polynucleotides of the invention as chromosomal markers.

#### EXAMPLE 18

##### Effect of Glucose Concentration and Hypoxia on VEGF, VEGF-B and VEGF-C mRNA levels in C6 Glioblastoma Cells

Confluent cultures of C6 cells (ATCC CCL 107) were grown on 10 cm diameter tissue culture plates containing 2.5 ml of DMEM and 5% fetal calf serum plus antibiotics. The cultures were exposed for 16 hours to normoxia in a normal cell culture incubator containing 5% CO<sub>2</sub> (FIG. 18: lanes marked -) or hypoxia (FIG. 18: lanes marked +) by closing the culture plates in an airtight glass chamber and burning a piece of wood inside until the flame was extinguished due to lack of oxygen. Polyadenylated RNA was isolated (as in the other examples), and 8 micrograms of the RNA was electrophoresed and blot-hybridized with a mixture of the VEGF, VEGF-B and VEGF-C probes (see FIG. 12). The results show that hypoxia strongly induces VEGF-A mRNA expression (compare lanes - and +), both in low and high glucose, but has no significant effect on the VEGF-B mRNA levels. The VEGF-C mRNA isolated from hypoxic cells runs slightly faster in gel electrophoresis and an extra band of faster mobility can be seen below the upper mRNA band. This observation suggests that hypoxia affects VEGF-C RNA processing. One explanation for this observation is that VEGF-C mRNA splicing is altered, affecting the VEGF-C open reading frame and resulting in an alternative VEGF-C protein being produced by hypoxic cells. Such alternative forms of VEGF-C and VEGF-C-encoding polynucleotides are contemplated as an aspect of the invention.

Deposit of Biological Materials: Plasmid FLT4-L has been deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Dr., Rockville Md. 20852 (USA), pursuant to the provisions of the Budapest Treaty, and has been assigned a deposit date of Jul. 24, 1995 and ATCC accession number 97231.

While the present invention has been described in terms of specific embodiments, it is understood that variations and modifications will occur to those in the art. Accordingly, only such limitations as appear in the appended claims should be placed on the invention.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 45

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TGTCTCGCT GTCCTTGCT

20

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 70 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ACATGCATGC CACCATGCAG CGGGGGCGCG CGCTGTGCCT GCGACTGTGG CTCTGCCTGG  
GACTCCTGGA

60

70

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ACATGCATGC CCGCGCGGTC ATCC

24

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CGGAATTCCC CATGACCCCA AC

22

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

-continued

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CCATCGATGG ATCCTACCTG AAGCCGCTTT CTT

33

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ATTAGGTGA CACTATA

17

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CCATCGATGG ATCCCGATGC TGCTTAGTAG CTGT

34

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Pro	Met	Thr	Pro	Thr	Thr	Tyr	Lys	Gly	Ser	Val	Asp	Asn	Gln	Thr	Asp
1			5					10						15	
Ser	Gly	Met	Val	Leu	Ala	Ser	Glu	Glu	Phe	Glu	Gln	Ile	Glu	Ser	Arg
		20				25			30						
His	Arg	Gln	Glu	Ser	Gly	Phe	Arg								
		35			40										

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CTGGAGTCGA CTTGGCGGAC T

21

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 60 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single

-continued

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CGCGGATCCC TAGTGATGGT GATGGTGATG TCTACCTTCG ATCATGCTGC CCTTATCCTC 60

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CCCAAGCTTG GATCCAAGTG GCTACTCCAT GACC 14

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GTTCGCTGTG ATGTGCACCA 20

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Xaa Glu Glu Thr Ile Lys Phe Ala Ala Ala His Tyr Asn Thr Glu Ile  
1 5 10 15  
Leu Lys

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GCAGARGARA CNATHAA 17

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

-continued

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Glu Glu Thr Ile Lys  
1 5

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 18 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GCAYTTNARD ATYTCNGT

18

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 5 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Thr Glu Ile Leu Lys  
1 5

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 22 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

ATTGCTGCA GCACACTACA AC

22

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 19 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TCNGTGTGT AGTGTGCTG

19

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 7 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

-continued-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Ala Ala His Tyr Asn Thr Glu  
1 5

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 20 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

TAATACGACT CACTATAGGG

20

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 24 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GTTGTAGTGT GCTGCAGCGA ATTT

24

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 8 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Lys Phe Ala Ala Ala His Tyr Asn  
1 5

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 21 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

TCACTATAGG GAGACCCAAG C

21

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 219 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

-continued

TCACTATAGG GAGACCCAAG CTGGTACCG AGCTCGGATC CACTAGTAAC GCGCGCCAGT 60  
GTGGTGGAAT TCGACGAACT CATGACTGTA CTCTACCCAG AATATTGGAA AATGTACAAG 120  
TGTCAGCTAA GGCAAGGAGG CTGGCAACAT AACAGAGAAC AGGCCAACCT CAACTCAAGG 180  
ACAGAAGAGA CTATAAAATT CGCTGCAGCA CACTACAAC 219

## (2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 18 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

ACAGAGAACA GGCCAACC 18

## (2) INFORMATION FOR SEQ ID NO:27:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 19 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

TCTAGCATTT AGGTGACAC 19

## (2) INFORMATION FOR SEQ ID NO:28:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 25 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

AAGAGACTAT AAAATTCGCT GCAGC 25

## (2) INFORMATION FOR SEQ ID NO:29:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

CCCTCTAGAT GCATGCTCGA 20

## (2) INFORMATION FOR SEQ ID NO:30:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 24 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)



-continued-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GTGTAGTGT GCTGCAGCGA ATTT

24

(2) INFORMATION FOR SEQ ID NO:31:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 21 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

TCACTATAGG GAGACCCAAG C

21

(2) INFORMATION FOR SEQ ID NO:32:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1140 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: CDS  
 (B) LOCATION: 37..1086

(ix) FEATURE:

- (A) NAME/KEY: mat\_peptide  
 (B) LOCATION: 136..1086

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

GAGCAGTTAC GGCTGTGTC CAGTGTAGAT GAACTC ATG ACT GTA CTC TAC CCA  
 Met Thr Val Leu Tyr Pro  
 -33 -30

54

GAA TAT TGG AAA ATG TAC AAG TGT CAG CTA AGG AAA GGA GGC TGG CAA  
 Gly Tyr Trp Lys Met Tyr Lys Cys Gln Leu Arg Lys Gly Gly Trp Gln  
 -25 -20 -15

102

CAT AAC AGA GAA CAG GCC AAC CTC AAC TCA AGG ACA GAA GAG ACT ATA  
 His Asn Arg Glu Gln Ala Asn Leu Asn Ser Arg Thr Glu Glu Thr Ile  
 -10 -5 1 5

150

AAA TTT GCT GCA GCA CAT TAT AAT ACA GAG ATC TTG AAA AGT ATT GAT  
 Lys Phe Ala Ala Ala His Tyr Asn Thr Glu Ile Leu Lys Ser Ile Asp  
 10 15 20

198

AAT GAG TGG AGA AAG ACT CAA TGC ATG CCA CGG GAG GTG TGT ATA GAT  
 Asn Glu Trp Arg Lys Thr Gln Cys Met Pro Arg Glu Val Cys Ile Asp  
 25 30 35

246

GTG GGG AAG GAG TTT GGA GTC GCG ACA AAC ACC TTC TTT AAA CCT CCA  
 Val Gly Lys Glu Phe Gly Val Ala Thr Asn Thr Phe Phe Lys Pro Pro  
 40 45 50

294

TGT GTC TCC GTC TAC AGA TGT GGG GGT TGC TGC AAT AGT GAG GGG CTG  
 Cys Val Ser Val Tyr Arg Cys Gly Gly Cys Cys Asn Ser Glu Gly Leu  
 55 60 65

342

CAG TGC ATG AAC ACC AGC ACG AGC TAC CTC AGC AAG ACG TTA TTT GAA  
 Gln Cys Met Asn Thr Ser Thr Ser Tyr Leu Ser Lys Thr Leu Phe Glu  
 70 75 80 85

390

ATT ACA GTG CCT CTC TCT CAA GGC CCC AAA CCA GTA ACA ATC AGT TTT  
 Ile Thr Val Pro Leu Ser Gln Gly Pro Lys Pro Val Thr Ile Ser Phe  
 90 95 100

438

GCC AAT CAC ACT TCC TGC CGA TGC ATG TCT AAA CTG GAT GTT TAC AGA  
 Ala Asn His Thr Ser Cys Arg Cys Met Ser Lys Leu Asp Val Tyr Arg

486

-continued

105	110	115	
CAA GTT CAT TCC ATT ATT AGA CGT TCC CTG CCA GCA ACA CTA CCA CAG Gln Val His Ser Ile Ile Arg Arg Ser Leu Pro Ala Thr Leu Pro Gln 120 125 130			534
TGT CAG GCA GCG AAC AAG ACC TGC CCC ACC AAT TAC ATG TGG AAT AAT Cys Gln Ala Ala Asn Lys Thr Cys Pro Thr Asn Tyr Met Trp Asn Asn 135 140 145			582
CAC ATC TGC AGA TGC CTG GCT CAG GAA GAT TTT ATG TTT TCC TCG GAT His Ile Cys Arg Cys Leu Ala Gln Glu Asp Phe Met Phe Ser Ser Asp 150 155 160 165			630
GCT GGA GAT GAC TCA ACA GAT GGA TTC CAT GAC ATC TGT GGA CCA AAC Ala Gly Asp Asp Ser Thr Asp Gly Phe His Asp Ile Cys Gly Pro Asn 170 175 180			678
AAG GAG CTG GAT GAA GAG ACC TGT CAG TGT GTC TGC AGA GCG GGG CTT Lys Glu Leu Asp Glu Glu Thr Cys Gln Cys Val Cys Arg Ala Gly Leu 185 190 195			726
CGG CCT GCC AGC TGT GGA CCC CAC AAA GAA CTA GAC AGA AAC TCA TGC Arg Pro Ala Ser Cys Gly Pro His Lys Glu Leu Asp Arg Asn Ser Cys 200 205 210			774
CAG TGT GTC TGT AAA AAC AAA CTC TTC CCC AGC CAA TGT GGG GCC AAC Gln Cys Val Cys Lys Asn Lys Leu Phe Pro Ser Gln Cys Gly Ala Asn 215 220 225			822
CGA GAA TTT GAT GAA AAC ACA TGC CAG TGT GTA TGT AAA AGA ACC TGC Arg Glu Phe Asp Glu Asn Thr Cys Gln Cys Val Cys Lys Arg Thr Cys 230 235 240 245			870
CCC AGA AAT CAA CCC CTA AAT CCT GGA AAA TGT GCC TGT GAA TGT ACA Pro Arg Asn Gln Pro Leu Asn Pro Gly Lys Cys Ala Cys Glu Cys Thr 250 255 260			918
GAA AGT CCA CAG AAA TGC TTG TTA AAA GGA AAG AAG TTC CAC CAC CAA Glu Ser Pro Gln Lys Cys Leu Leu Lys Gly Lys Lys Phe His His Gln 265 270 275			966
ACA TGC AGC TGT TAC AGA CGG CCA TGT ACG AAC CGC CAG AAG GCT TGT Thr Cys Ser Cys Tyr Arg Arg Pro Cys Thr Asn Arg Gln Lys Ala Cys 280 285 290			1014
GAG CCA GGA TTT TCA TAT AGT GAA GAA GTG TGT CGT TGT GTC CCT TCA Glu Pro Gly Phe Ser Tyr Ser Glu Glu Val Cys Arg Cys Val Pro Ser 295 300 305			1062
TAT TGG AAA AGA CCA CAA ATG AGC TAAGATTGTA CTGTTTTCCTA GTTCATCGAT Tyr Trp Lys Arg Pro Gln Met Ser 310 315			1116
TTTCTATTAT GGAAACTGT GTTG			1140

## (2) INFORMATION FOR SEQ ID NO:33:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 350 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

Met Thr Val Leu Tyr Pro Glu Tyr Trp Lys Met Tyr Lys Cys Gln Leu  
-33 -30 -25 -20

Arg Lys Gly Gly Trp Gln His Asn Arg Glu Gln Ala Asn Leu Asn Ser  
-15 -10 -5

Arg Thr Glu Glu Thr Ile Lys Phe Ala Ala Ala His Tyr Asn Thr Glu  
1 5 10 15

Ile Leu Lys Ser Ile Asp Asn Glu Trp Arg Lys Thr Gln Cys Met Pro  
20 25 30

-continued

Arg Glu Val Cys Ile Asp Val Gly Lys Glu Phe Gly Val Ala Thr Asn  
 35 40 45  
 Thr Phe Phe Lys Pro Pro Cys Val Ser Val Tyr Arg Cys Gly Gly Cys  
 50 55 60  
 Cys Asn Ser Glu Gly Leu Gln Cys Met Asn Thr Ser Thr Ser Tyr Leu  
 65 70 75  
 Ser Lys Thr Leu Phe Glu Ile Thr Val Pro Leu Ser Gln Gly Pro Lys  
 80 85 90 95  
 Pro Val Thr Ile Ser Phe Ala Asn His Thr Ser Cys Arg Cys Met Ser  
 100 105 110  
 Lys Leu Asp Val Tyr Arg Gln Val His Ser Ile Ile Arg Arg Ser Leu  
 115 120 125  
 Pro Ala Thr Leu Pro Gln Cys Gln Ala Ala Asn Lys Thr Cys Pro Thr  
 130 135 140  
 Asn Tyr Met Trp Asn Asn His Ile Cys Arg Cys Leu Ala Gln Glu Asp  
 145 150 155  
 Phe Met Phe Ser Ser Asp Ala Gly Asp Asp Ser Thr Asp Gly Phe His  
 160 165 170 175  
 Asp Ile Cys Gly Pro Asn Lys Glu Leu Asp Glu Glu Thr Cys Gln Cys  
 180 185 190  
 Val Cys Arg Ala Gly Leu Arg Pro Ala Ser Cys Gly Pro His Lys Glu  
 195 200 205  
 Leu Asp Arg Asn Ser Cys Gln Cys Val Cys Lys Asn Lys Leu Phe Pro  
 210 215 220  
 Ser Gln Cys Gly Ala Asn Arg Glu Phe Asp Glu Asn Thr Cys Gln Cys  
 225 230 235  
 Val Cys Lys Arg Thr Cys Pro Arg Asn Gln Pro Leu Asn Pro Gly Lys  
 240 245 250 255  
 Cys Ala Cys Glu Cys Thr Glu Ser Pro Gln Lys Cys Leu Leu Lys Gly  
 260 265 270  
 Lys Lys Phe His His Gln Thr Cys Ser Cys Tyr Arg Arg Pro Cys Thr  
 275 280 285  
 Asn Arg Gln Lys Ala Cys Glu Pro Gly Phe Ser Tyr Ser Glu Glu Val  
 290 295 300  
 Cys Arg Cys Val Pro Ser Tyr Trp Lys Arg Pro Gln Met Ser  
 305 310 315

## (2) INFORMATION FOR SEQ ID NO:34:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 22 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

TGAGTGATTGTAGCTGCTGTG

22

## (2) INFORMATION FOR SEQ ID NO:35:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 22 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

-continued

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

TATTGCAGCAACCCCCACATCT

22

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 196 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: Not Relevant
- (D) TOPOLOGY: Not Relevant

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

```

Met Arg Thr Trp Ala Cys Leu Leu Leu Gly Cys Gly Tyr Leu Ala
1      5      10      15
His Ala Leu Ala Glu Glu Ala Glu Ile Pro Arg Glu Leu Ile Glu Arg
20     25     30
Leu Ala Arg Ser Gln Ile His Ser Ile Arg Asp Leu Gln Arg Leu Leu
35     40     45
Glu Ile Asp Ser Val Gly Ala Glu Asp Ala Leu Glu Thr Ser Leu Arg
50     55     60
Ala His Gly Ser His Ala Ile Asn His Val Pro Glu Lys Arg Pro Val
65     70     75     80
Pro Ile Arg Arg Lys Arg Ser Ile Glu Glu Ala Ile Pro Ala Val Cys
85     90     95
Lys Thr Arg Thr Val Ile Tyr Glu Ile Pro Arg Ser Gln Val Asp Pro
100    105    110
Thr Ser Ala Asn Phe Leu Ile Trp Pro Pro Cys Val Glu Val Lys Arg
115    120    125
Cys Thr Gly Cys Cys Asn Thr Ser Ser Val Lys Cys Gln Pro Ser Arg
130    135    140
Val His His Arg Ser Val Lys Val Ala Lys Val Glu Tyr Val Arg Lys
145    150    155    160
Lys Pro Lys Leu Lys Glu Val Gln Val Arg Leu Glu Glu His Leu Glu
165    170    175
Cys Ala Cys Ala Thr Ser Asn Leu Asn Pro Asp His Arg Glu Glu Glu
180    185    190
Thr Asp Val Arg
195

```

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 241 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: Not Relevant
- (D) TOPOLOGY: Not Relevant

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

```

Met Asn Arg Cys Trp Ala Leu Phe Leu Ser Leu Cys Cys Tyr Leu Arg
1      5      10      15
Leu Val Ser Ala Glu Gly Asp Pro Ile Pro Glu Glu Leu Tyr Glu Met
20     25     30
Leu Ser Asp His Ser Ile Arg Ser Phe Asp Asp Leu Gln Arg Leu Leu
35     40     45

```

-continued-

His Gly Asp Pro Gly Glu Glu Asp Gly Ala Glu Leu Asp Leu Asn Met  
 50 55 60  
 Thr Arg Ser His Ser Gly Gly Glu Leu Glu Ser Leu Ala Arg Gly Arg  
 65 70 75 80  
 Arg Ser Leu Gly Ser Leu Thr Ile Ala Glu Pro Ala Met Ile Ala Glu  
 85 90 95  
 Cys Lys Thr Arg Thr Glu Val Phe Glu Ile Ser Arg Arg Leu Ile Asp  
 100 105 110  
 Arg Thr Asn Ala Asn Phe Leu Val Trp Pro Pro Cys Val Glu Val Gln  
 115 120 125  
 Arg Cys Ser Gly Cys Cys Asn Asn Arg Asn Val Gln Cys Arg Pro Thr  
 130 135 140  
 Gln Val Gln Leu Arg Pro Val Gln Val Arg Lys Ile Glu Ile Val Arg  
 145 150 155 160  
 Lys Lys Pro Ile Phe Lys Lys Ala Thr Val Thr Leu Glu Asp His Leu  
 165 170 175  
 Ala Cys Lys Cys Glu Thr Val Ala Ala Ala Arg Pro Val Thr Arg Ser  
 180 185 190  
 Pro Gly Gly Ser Gln Glu Gln Arg Ala Lys Thr Pro Gln Thr Arg Val  
 195 200 205  
 Thr Ile Arg Thr Val Arg Val Arg Arg Pro Pro Lys Gly Lys His Arg  
 210 215 220  
 Lys Phe Lys His Thr His Asp Lys Thr Ala Leu Lys Glu Thr Leu Gly  
 225 230 235 240  
 Ala

## (2) INFORMATION FOR SEQ ID NO:38:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 149 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: Not Relevant
- (D) TOPOLOGY: Not Relevant

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

Met Pro Val Met Arg Leu Phe Pro Cys Phe Leu Gln Leu Leu Ala Gly  
 1 5 10 15  
 Leu Ala Leu Pro Ala Val Pro Pro Gln Gln Trp Ala Leu Ser Ala Gly  
 20 25 30  
 Asn Gly Ser Ser Glu Val Glu Val Val Pro Phe Gln Glu Val Trp Gly  
 35 40 45  
 Arg Ser Tyr Cys Arg Ala Leu Glu Arg Leu Val Asp Val Val Ser Glu  
 50 55 60  
 Tyr Pro Ser Glu Val Glu His Met Phe Ser Pro Ser Cys Val Ser Leu  
 65 70 75 80  
 Leu Arg Cys Thr Gly Cys Cys Gly Asp Glu Asn Leu His Cys Val Pro  
 85 90 95  
 Val Glu Thr Ala Asn Val Thr Met Gln Leu Leu Lys Ile Arg Ser Gly  
 100 105 110  
 Asp Arg Pro Ser Tyr Val Glu Leu Thr Phe Ser Gln His Val Arg Cys  
 115 120 125  
 Glu Cys Arg Pro Leu Arg Glu Lys Met Lys Pro Glu Arg Cys Gly Asp  
 130 135 140

-continued

Ala Val Pro Arg Arg  
145

## (2) INFORMATION FOR SEQ ID NO:39:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 170 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: Not Relevant
- (D) TOPOLOGY: Not Relevant

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

Met Pro Val Met Arg Leu Phe Pro Cys Phe Leu Gln Leu Leu Ala Gly  
1 5 10 15  
Leu Ala Leu Pro Ala Val Pro Pro Gln Gln Trp Ala Leu Ser Ala Gly  
20 25 30  
Asn Gly Ser Ser Glu Val Glu Val Val Pro Phe Gln Glu Val Trp Gly  
35 40 45  
Arg Ser Tyr Cys Arg Ala Leu Glu Arg Leu Val Asp Val Val Ser Glu  
50 55 60  
Tyr Pro Ser Glu Val Glu His Met Phe Ser Pro Ser Cys Val Ser Leu  
65 70 75 80  
Leu Arg Cys Thr Gly Cys Cys Gly Asp Glu Asn Leu His Cys Val Pro  
85 90 95  
Val Glu Thr Ala Asn Val Thr Met Gln Leu Leu Lys Ile Arg Ser Gly  
100 105 110  
Asp Arg Pro Ser Tyr Val Glu Leu Thr Phe Ser Gln His Val Arg Cys  
115 120 125  
Glu Cys Arg Pro Leu Arg Glu Lys Met Lys Pro Glu Arg Arg Arg Pro  
130 135 140  
Lys Gly Arg Gly Lys Arg Arg Arg Glu Lys Gln Arg Pro Thr Asp Cys  
145 150 155 160  
His Leu Cys Gly Asp Ala Val Pro Arg Arg  
165 170

## (2) INFORMATION FOR SEQ ID NO:40:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 147 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: Not Relevant
- (D) TOPOLOGY: Not Relevant

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

Met Asn Phe Leu Leu Ser Trp Val His Trp Ser Leu Ala Leu Leu Leu  
1 5 10 15  
Tyr Leu His His Ala Lys Trp Ser Gln Ala Ala Pro Met Ala Glu Gly  
20 25 30  
Gly Gly Gln Asn His His Glu Val Val Lys Phe Met Asp Val Tyr Gln  
35 40 45  
Arg Ser Tyr Cys His Pro Ile Glu Thr Leu Val Asp Ile Phe Gln Glu  
50 55 60  
Tyr Pro Asp Glu Ile Glu Tyr Ile Phe Lys Pro Ser Cys Val Pro Leu  
65 70 75 80  
Met Arg Cys Gly Gly Cys Cys Asn Asp Glu Gly Leu Glu Cys Val Pro  
85 90 95

-continued

Thr Glu Glu Ser Asn Ile Thr Met Gln Ile Met Arg Ile Lys Pro His  
 100 105 110  
 Gln Gly Gln His Ile Gly Glu Met Ser Phe Leu Gln His Asn Lys Cys  
 115 120 125  
 Glu Cys Arg Pro Lys Lys Asp Arg Ala Arg Gln Glu Lys Cys Asp Lys  
 130 135 140  
 Pro Arg Arg  
 145

## (2) INFORMATION FOR SEQ ID NO:41:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 191 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: Not Relevant
- (D) TOPOLOGY: Not Relevant

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

Met Asn Phe Leu Leu Ser Trp Val His Trp Ser Leu Ala Leu Leu Leu  
 1 5 10 15  
 Tyr Leu His His Ala Lys Trp Ser Gln Ala Ala Pro Met Ala Glu Gly  
 20 25 30  
 Gly Gly Gln Asn His His Glu Val Val Lys Phe Met Asp Val Tyr Gln  
 35 40 45  
 Arg Ser Tyr Cys His Pro Ile Glu Thr Leu Val Asp Ile Phe Gln Glu  
 50 55 60  
 Tyr Pro Asp Glu Ile Glu Tyr Ile Phe Lys Pro Ser Cys Val Pro Leu  
 65 70 75 80  
 Met Arg Cys Gly Gly Cys Cys Asn Asp Glu Gly Leu Glu Cys Val Pro  
 85 90 95  
 Thr Glu Glu Ser Asn Ile Thr Met Gln Ile Met Arg Ile Lys Pro His  
 100 105 110  
 Gln Gly Gln His Ile Gly Glu Met Ser Phe Leu Gln His Asn Lys Cys  
 115 120 125  
 Glu Cys Arg Pro Lys Lys Asp Arg Ala Arg Gln Glu Asn Pro Cys Gly  
 130 135 140  
 Pro Cys Ser Glu Arg Arg Lys His Leu Phe Val Gln Asp Pro Gln Thr  
 145 150 155 160  
 Cys Lys Cys Ser Cys Lys Asn Thr Asp Ser Arg Cys Lys Ala Arg Gln  
 165 170 175  
 Leu Glu Leu Asn Glu Arg Thr Cys Arg Cys Asp Lys Pro Arg Arg  
 180 185 190

## (2) INFORMATION FOR SEQ ID NO:42:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 215 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: Not Relevant
- (D) TOPOLOGY: Not Relevant

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

Met Asn Phe Leu Leu Ser Trp Val His Trp Ser Leu Ala Leu Leu Leu  
 1 5 10 15  
 Tyr Leu His His Ala Lys Trp Ser Gln Ala Ala Pro Met Ala Glu Gly

-continued

20					25					30					
Gly	Gly	Gln	Asn	His	His	Glu	Val	Val	Lys	Phe	Met	Asp	Val	Tyr	Gln
		35					40					45			
Arg	Ser	Tyr	Cys	His	Pro	Ile	Glu	Thr	Leu	Val	Asp	Ile	Phe	Gln	Glu
	50					55					60				
Tyr	Pro	Asp	Glu	Ile	Glu	Tyr	Ile	Phe	Lys	Pro	Ser	Cys	Val	Pro	Leu
	65				70					75				80	
Met	Arg	Cys	Gly	Gly	Cys	Cys	Asn	Asp	Glu	Gly	Leu	Glu	Cys	Val	Pro
			85						90					95	
Thr	Glu	Glu	Ser	Asn	Ile	Thr	Met	Gln	Ile	Met	Arg	Ile	Lys	Pro	His
			100					105					110		
Gln	Gly	Gln	His	Ile	Gly	Glu	Met	Ser	Phe	Leu	Gln	His	Asn	Lys	Cys
		115					120					125			
Glu	Cys	Arg	Pro	Lys	Lys	Asp	Arg	Ala	Arg	Gln	Glu	Lys	Lys	Ser	Val
	130					135					140				
Arg	Gly	Lys	Gly	Lys	Gly	Gln	Lys	Arg	Lys	Arg	Lys	Lys	Ser	Arg	Tyr
	145				150				155					160	
Lys	Ser	Trp	Ser	Val	Pro	Cys	Gly	Pro	Cys	Ser	Glu	Arg	Arg	Lys	His
			165					170						175	
Leu	Phe	Val	Gln	Asp	Pro	Gln	Thr	Cys	Lys	Cys	Ser	Cys	Lys	Asn	Thr
			180				185						190		
Asp	Ser	Arg	Cys	Lys	Ala	Arg	Gln	Leu	Glu	Leu	Asn	Glu	Arg	Thr	Cys
	195					200						205			
Arg	Cys	Asp	Lys	Pro	Arg	Arg									
	210				215										

## (2) INFORMATION FOR SEQ ID NO:43:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 232 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: Not Relevant
- (D) TOPOLOGY: Not Relevant

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

Met	Asn	Phe	Leu	Leu	Ser	Trp	Val	His	Trp	Ser	Leu	Ala	Leu	Leu	Leu
1			5						10				15		
Tyr	Leu	His	His	Ala	Lys	Trp	Ser	Gln	Ala	Ala	Pro	Met	Ala	Glu	Gly
		20						25				30			
Gly	Gly	Gln	Asn	His	His	Glu	Val	Val	Lys	Phe	Met	Asp	Val	Tyr	Gln
		35					40					45			
Arg	Ser	Tyr	Cys	His	Pro	Ile	Glu	Thr	Leu	Val	Asp	Ile	Phe	Gln	Glu
	50					55					60				
Tyr	Pro	Asp	Glu	Ile	Glu	Tyr	Ile	Phe	Lys	Pro	Ser	Cys	Val	Pro	Leu
	65				70				75					80	
Met	Arg	Cys	Gly	Gly	Cys	Cys	Asn	Asp	Glu	Gly	Leu	Glu	Cys	Val	Pro
			85						90					95	
Thr	Glu	Glu	Ser	Asn	Ile	Thr	Met	Gln	Ile	Met	Arg	Ile	Lys	Pro	His
		100					105					110			
Gln	Gly	Gln	His	Ile	Gly	Glu	Met	Ser	Phe	Leu	Gln	His	Asn	Lys	Cys
		115					120					125			
Glu	Cys	Arg	Pro	Lys	Lys	Asp	Arg	Ala	Arg	Gln	Glu	Lys	Lys	Ser	Val
	130					135					140				
Arg	Gly	Lys	Gly	Lys	Gly	Gln	Lys	Arg	Lys	Arg	Lys	Lys	Ser	Arg	Tyr



-continued

145	150	155	160
Lys Ser Trp Ser Val Tyr Val Gly Ala Arg Cys Cys Leu Met Pro Trp			
165		170	175
Ser Leu Pro Gly Pro His Pro Cys Gly Pro Cys Ser Glu Arg Arg Lys			
180	185		190
His Leu Phe Val Gln Asp Pro Gln Thr Cys Lys Cys Ser Cys Lys Asn			
195	200		205
Thr Asp Ser Arg Cys Lys Ala Arg Gln Leu Glu Leu Asn Glu Arg Thr			
210	215		220
Cys Arg Cys Asp Lys Pro Arg Arg			
225	230		

## (2) INFORMATION FOR SEQ ID NO:44:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1997 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

- (ix) FEATURE:  
 (A) NAME/KEY: CDS  
 (B) LOCATION: 352..1608

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

CCCCCCCCCG CTCTCCAAAA AGCTACACCG ACGCGGACCG CGGCGGCGTC CTCCTCGCC	60
CTCGCTTCAC CTCGCGGGCT CCGAATGCGG GGAGCTCGGA TGTCCGGTTT CCTGTGAGGC	120
TTTACCTGA CACCCGCCGC GTTCCCGCG CACTGGCTGG GAGGGCGCCC TGCAAAAGTTG	180
GGAACGCGGA GCGCCGGACC CGCTCCCGCC GCCTCGGGCT CGCCAGGGG GGGTCGCGCG	240
GAGGAGCCCG GGGAGAGGG ACCAGGAGGG GCGCGCGGCC TCGCAGGGGC GCGCGCGCCC	300
CCACCCCTGC CCGCGCCAGC GGACCGGTCC CCCACCCCG GTCTTCCAC C ATG CAC	357
	Met His
TTG CTG GGC TTC TTC TCT GTG GCG TGT TCT CTG CTC GCC GCT GCG CTG	405
Leu Leu Gly Phe Phe Ser Val Ala Cys Ser Leu Leu Ala Ala Leu	
5 10 15	
CTC CCG GGT CCT CSC GAG GCG CCC GCC GCC GCC GCC TTC GAG TCC	453
Leu Pro Gly Pro Arg Glu Ala Pro Ala Ala Ala Ala Phe Glu Ser	
20 25 30	
GGA CTC GAC CTC TCG GAC GCG GAG CCC GAC GCG GGC GAG GCC ACG GCT	501
Gly Leu Asp Leu Ser Asp Ala Glu Pro Asp Ala Gly Glu Ala Thr Ala	
35 40 45 50	
TAT GCA AGC AAA GAT CTG GAG GAG CAG TTA CGG TCT GTG TCC AGT GTA	549
Tyr Ala Ser Lys Asp Leu Glu Glu Gln Leu Arg Ser Val Ser Ser Val	
55 60 65	
GAT GAA CTC ATG ACT GTA CTC TAC CCA GAA TAT TGG AAA ATG TAC AAG	597
Asp Glu Leu Met Thr Val Leu Tyr Pro Glu Tyr Trp Lys Met Tyr Lys	
70 75 80	
TGT CAG CTA AGG AAA GGA GGC TGG CAA CAT AAC AGA GAA CAG GCC AAC	645
Cys Gln Leu Arg Lys Gly Gly Trp Gln His Asn Arg Glu Gln Ala Asn	
85 90 95	
CTC AAC TCA AGG ACA GAA GAG ACT ATA AAA TTT GCT GCA GCA CAT TAT	693
Leu Asn Ser Arg Thr Glu Glu Thr Ile Lys Phe Ala Ala Ala His Tyr	
100 105 110	
AAT ACA GAG ATC TTG AAA AGT ATT GAT AAT GAG TGG AGA AAG ACT CAA	741
Asn Thr Glu Ile Leu Lys Ser Ile Asp Asn Glu Trp Arg Lys Thr Gln	
115 120 125 130	

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TGC ATG CCA CGG GAG GTG TGT ATA GAT GTG GGG AAG GAG TTT GGA GTC Cys Met Pro Arg Glu Val Cys Ile Asp Val Gly Lys Glu Phe Gly Val 135 140 145	789
GCG ACA AAC ACC TTC TTT AAA CCT CCA TGT GTG TCC GTC TAC AGA TGT Ala Thr Asn Thr Phe Phe Lys Pro Pro Cys Val Ser Val Tyr Arg Cys 150 155 160	837
GGG GGT TGC TGC AAT AGT GAG GGG CTG CAG TGC ATG AAC ACC AGC ACG Gly Gly Cys Cys Asn Ser Glu Gly Leu Gln Cys Met Asn Thr Ser Thr 165 170 175	885
AGC TAC CTC AGC AAG ACG TTA TTT GAA ATT ACA GTG CCT CTC TCT CAA Ser Tyr Leu Ser Lys Thr Leu Phe Glu Ile Thr Val Pro Leu Ser Gln 180 185 190	933
GGC CCC AAA CCA GTA ACA ATC AGT TTT GCC AAT CAC ACT TCC TGC CGA Gly Pro Lys Pro Val Thr Ile Ser Phe Ala Asn His Thr Ser Cys Arg 195 200 205 210	981
TGC ATG TCT AAA CTG GAT GTT TAC AGA CAA GTT CAT TCC ATT ATT AGA Cys Met Ser Lys Leu Asp Val Tyr Arg Gln Val His Ser Ile Ile Arg 215 220 225	1029
CGT TCC CTG CCA GCA ACA CTA CCA CAG TGT CAG GCA GCG AAC AAG ACC Arg Ser Leu Pro Ala Thr Leu Pro Gln Cys Gln Ala Ala Asn Lys Thr 230 235 240	1077
TGC CCC ACC AAT TAC ATG TGG AAT AAT CAC ATC TGC AGA TGC CTG GCT Cys Pro Thr Asn Tyr Met Trp Asn Asn His Ile Cys Arg Cys Leu Ala 245 250 255	1125
CAG GAA GAT TTT ATG TTT TCC TCG GAT GCT GGA GAT GAC TCA ACA GAT Gln Glu Asp Phe Met Phe Ser Ser Asp Ala Gly Asp Asp Ser Thr Asp 260 265 270	1173
GGA TTC CAT GAC ATC TGT GGA CCA AAC AAG GAG CTG GAT GAA GAG ACC Gly Phe His Asp Ile Cys Gly Pro Asn Lys Glu Leu Asp Glu Glu Thr 275 280 285 290	1221
TGT CAG TGT GTC TGC AGA GCG GGG CTT CGG CCT GCC AGC TGT GGA CCC Cys Gln Cys Val Cys Arg Ala Gly Leu Arg Pro Ala Ser Cys Gly Pro 295 300 305	1269
CAC AAA GAA CTA GAC AGA AAC TCA TGC CAG TGT GTC TGT AAA AAC AAA His Lys Glu Leu Asp Arg Asn Ser Cys Gln Cys Val Cys Lys Asn Lys 310 315 320	1317
CTC TTC CCC AGC CAA TGT GGG GCC AAC CGA GAA TTT GAT GAA AAC ACA Leu Phe Pro Ser Gln Cys Gly Ala Asn Arg Glu Phe Asp Glu Asn Thr 325 330 335	1365
TGC CAG TGT GTA TGT AAA AGA ACC TGC CCC AGA AAT CAA CCC CTA AAT Cys Gln Cys Val Cys Lys Arg Thr Cys Pro Arg Asn Gln Pro Leu Asn 340 345 350	1413
CCT GGA AAA TGT GCC TGT GAA TGT ACA GAA AGT CCA CAG AAA TGC TTG Pro Gly Lys Cys Ala Cys Glu Cys Thr Glu Ser Pro Gln Lys Cys Leu 355 360 365 370	1461
TTA AAA GGA AAG AAG TTC CAC CAC CAA ACA TGC AGC TGT TAC AGA CGG Leu Lys Gly Lys Lys Phe His His Gln Thr Cys Ser Cys Tyr Arg Arg 375 380 385	1509
CCA TGT ACG AAC CGC CAG AAG GCT TGT GAG CCA GGA TTT TCA TAT AGT Pro Cys Thr Asn Arg Gln Lys Ala Cys Glu Pro Gly Phe Ser Tyr Ser 390 395 400	1557
GAA GAA GTG TGT CGT TGT GTC CCT TCA TAT TGG AAA AGA CCA CAA ATG Glu Glu Val Cys Arg Cys Val Pro Ser Tyr Trp Lys Arg Pro Gln Met 405 410 415	1605
AGC TAAGATTGTA CTGTTTTCCA GTTCATCGAT TTTCTATTAT GGAAAACGTG Ser	1658
GTTCACACAG TAGAACTGTC TGTGAACAGA GAGACCCCTG TGGGTCCATG CTAACAAAGA	1718
CAAAAGTCTG TCTTTCCTGA ACCATGTGGA TAACTTTACA GAAATGGACT GGAGTCATC	1778

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TGCAAAAGGC CTCTTGTAAG GACTGGTTTT CTGCCAATGA CCAACAGCC AAGATTTTC 1838  
 TCTTGATGATT TCTTTAAAG AATGACTATA TAATTTATTT CCACTAAAAA TATTGTTTCT 1898  
 GCATTCAATT TTATAGCAAC AACAATTGGT AAAACTCACT GTGATCAATA TTTTATATC 1958  
 ATGCAAAATA TGTTTAAAT AAAATGAAAA TTGTATTAT 1997

## (2) INFORMATION FOR SEQ ID NO:45:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 419 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

Met His Leu Leu Gly Phe Phe Ser Val Ala Cys Ser Leu Leu Ala Ala  
 1 5 10 15  
 Ala Leu Leu Pro Gly Pro Arg Glu Ala Pro Ala Ala Ala Ala Phe  
 20 25 30  
 Glu Ser Gly Leu Asp Leu Ser Asp Ala Glu Pro Asp Ala Gly Glu Ala  
 35 40 45  
 Thr Ala Tyr Ala Ser Lys Asp Leu Glu Glu Gln Leu Arg Ser Val Ser  
 50 55 60  
 Ser Val Asp Glu Leu Met Thr Val Leu Tyr Pro Glu Tyr Trp Lys Met  
 65 70 75 80  
 Tyr Lys Cys Gln Leu Arg Lys Gly Gly Trp Gln His Asn Arg Glu Gln  
 85 90 95  
 Ala Asn Leu Asn Ser Arg Thr Glu Glu Thr Ile Lys Phe Ala Ala Ala  
 100 105 110  
 His Tyr Asn Thr Glu Ile Leu Lys Ser Ile Asp Asn Glu Trp Arg Lys  
 115 120 125  
 Thr Gln Cys Met Pro Arg Glu Val Cys Ile Asp Val Gly Lys Glu Phe  
 130 135 140  
 Gly Val Ala Thr Asn Thr Phe Phe Lys Pro Pro Cys Val Ser Val Tyr  
 145 150 155 160  
 Arg Cys Gly Gly Cys Cys Asn Ser Glu Gly Leu Gln Cys Met Asn Thr  
 165 170 175  
 Ser Thr Ser Tyr Leu Ser Lys Thr Leu Phe Glu Ile Thr Val Pro Leu  
 180 185 190  
 Ser Gln Gly Pro Lys Pro Val Thr Ile Ser Phe Ala Asn His Thr Ser  
 195 200 205  
 Cys Arg Cys Met Ser Lys Leu Asp Val Tyr Arg Gln Val His Ser Ile  
 210 215 220  
 Ile Arg Arg Ser Leu Pro Ala Thr Leu Pro Gln Cys Gln Ala Ala Asn  
 225 230 235 240  
 Lys Thr Cys Pro Thr Asn Tyr Met Trp Asn Asn His Ile Cys Arg Cys  
 245 250 255  
 Leu Ala Gln Glu Asp Phe Met Phe Ser Ser Asp Ala Gly Asp Asp Ser  
 260 265 270  
 Thr Asp Gly Phe His Asp Ile Cys Gly Pro Asn Lys Glu Leu Asp Glu  
 275 280 285  
 Glu Thr Cys Gln Cys Val Cys Arg Ala Gly Leu Arg Pro Ala Ser Cys  
 290 295 300  
 Gly Pro His Lys Glu Leu Asp Arg Asn Ser Cys Gln Cys Val Cys Lys

-continued

105	310	315	320
Asn Lys Leu Phe Pro Ser Gln Cys Gly Ala Asn Arg Glu Phe Asp Glu			
325		330	335
Asn Thr Cys Gln Cys Val Cys Lys Arg Thr Cys Pro Arg Asn Gln Pro			
340	345		350
Leu Asn Pro Gly Lys Cys Ala Cys Glu Cys Thr Glu Ser Pro Gln Lys			
355	360	365	
Cys Leu Leu Lys Gly Lys Lys Phe His His Gln Thr Cys Ser Cys Tyr			
370	375	380	
Arg Arg Pro Cys Thr Asn Arg Gln Lys Ala Cys Glu Pro Gly Phe Ser			
385	390	395	400
Tyr Ser Glu Glu Val Cys Arg Cys Val Pro Ser Tyr Trp Lys Arg Pro			
	405	410	415
Gln Met Ser			

What is claimed is:

1. A purified and isolated nucleic acid comprising a nucleotide sequence that encodes a polypeptide that binds to human Flt4 receptor tyrosine kinase (Flt4), said polypeptide having an amino acid sequence comprising a portion of the amino acid sequence shown in SEQ ID NO: 33 effective to permit such binding, said nucleic acid lacking a nucleotide sequence that encodes the portion of the amino acid sequence shown in SEQ ID NO: 33 that has cysteine motifs of a Balbiani ring 3 protein.
2. A purified and isolated nucleic acid according to claim 1 wherein said polypeptide stimulates tyrosine phosphorylation of human Flt4.
3. A purified and isolated nucleic acid according to claim 2 wherein said polypeptide has an apparent molecular weight of about 23 kD as assessed by SDS polyacrylamide gel electrophoresis under reducing conditions.
4. A purified and isolated nucleic acid according to claim 2 wherein said polypeptide comprises an amino-terminal amino acid sequence set forth in SEQ ID NO: 13.
5. A purified and isolated nucleic acid according to claim 4 wherein said polypeptide comprises approximately 120 amino acids.
6. A purified and isolated nucleic acid according to claim 4 wherein amino terminal amino acids 2-through 18 of said polypeptide have an amino acid sequence identical to amino acids 2 through 18 set forth in SEQ ID NO: 13.
7. A purified and isolated nucleic acid according to claim 2 wherein said polypeptide comprises amino acids 1 to 120 of SEQ ID NO: 33.
8. A purified and isolated nucleic acid according to claim 1 wherein said polypeptide has an apparent molecular weight of about 32 kDa as assessed by SDS polyacrylamide gel electrophoresis under reducing conditions.
9. A nucleic acid according to claim 1 wherein said portion of the amino acid sequence shown in SEQ ID NO: 33 is a continuous portion that includes eight cysteines of SEQ ID NO: 33 that are conserved in human vascular endothelial growth factor (VEGF), human platelet derived growth factor A (PDGF-A), and human platelet derived growth factor B (PDGF-B), and excludes the carboxyl terminal portion of SEQ ID NO: 33 that contains cysteine motifs of a Balbiani ring 3 protein.
10. A nucleic acid according to claim 9 wherein said continuous portion has amino acid 1 of SEQ ID NO: 33 as its amino terminus.
11. A nucleic acid according to claim 1 wherein said portion of the amino acid sequence shown in SEQ ID NO: 33 is a continuous portion having amino acid 1 of SEQ ID NO: 33 as its amino terminal residue, and having as its carboxy terminal residue an amino acid between residues 119 and 126 of SEQ ID NO: 33.
12. A vector comprising a nucleic acid according to claim 1, wherein said vector lacks a nucleotide sequence that encodes the portion of the amino acid sequence shown in SEQ ID NO: 33 that has cysteine motifs of a Balbiani ring 3 protein.
13. A host cell transformed or transfected with a vector according to claim 12.
14. A method for producing a polypeptide that binds to the extracellular domain of human Flt4, comprising the steps of: growing a host cell according to claim 13 under conditions which permit expression by said host cell of a polypeptide that is encoded by said nucleic acid and that binds to the extracellular domain of human Flt4; and isolating said polypeptide from the host cell or the growth medium of the host cell.
15. A method according to claim 14 wherein said host cell is a mammalian host cell that secretes said polypeptide and wherein said isolating step comprises isolating said polypeptide from said growth medium.
16. A purified and isolated nucleic acid comprising a nucleotide sequence that encodes a polypeptide that binds human Flt4 receptor tyrosine kinase (Flt4), said polypeptide having an amino acid sequence comprising a continuous portion of the amino acid sequence shown in SEQ ID NO: 33 effective to permit such binding, said nucleic acid lacking a nucleotide sequence that encodes the carboxy-terminal portion of the amino acid sequence shown in SEQ ID NO: 33 beyond residue 125.
17. A purified and isolated nucleic acid according to claim 16 wherein said encoded polypeptide stimulates tyrosine phosphorylation of human Flt4.
18. A purified and isolated nucleic acid according to claim 16 wherein said nucleic acid lacks a nucleotide sequence that encodes the amino terminal portion of the amino acid sequence shown in SEQ ID NO: 33 that precedes residue 1.
19. An expression construct comprising the nucleic acid according to claim 18 operatively linked to an expression control sequence, said expression construct lacking a nucle-

otide sequence that encodes the carboxy-terminal portion of the amino acid sequence shown in SEQ ID NO:33 beyond residue 125.

20. A host cell transformed or transfected with the expression construct of claim 19.

21. A method for producing a polypeptide that binds to the extracellular domain of human Flt4 and stimulates tyrosine phosphorylation of Flt4, comprising the steps of:

growing a host cell according to claim 20 under conditions which permit expression in said host cell of a polypeptide encoded by said nucleic acid and isolating said polypeptide from the host cell or the growth medium of the host cell, wherein said polypeptide binds to the extracellular domain of human Flt4 and stimulates phosphorylation of Flt4.

22. A host cell transformed or transfected with a polynucleotide,

wherein said polynucleotide includes a strand containing a human nucleotide sequence that hybridizes to a DNA comprising the non-coding strand complementary to SEQ ID NO: 32, under the following hybridization conditions:

- (a) hybridization at 42° C. for 20 hours in a solution containing 50% formamide, 5×SSPE, 5×Denhardt's solution, 0.1% SDS and 0.1 mg/ml denatured salmon sperm DNA; and
- (b) washing the filter twice for thirty minutes at room temperature and twice for thirty minutes at 65° C. with a wash solution containing 1×SSC, and 0.1% SDS; and

wherein said host cell expresses a polypeptide encoded by said polynucleotide,

wherein said polypeptide has a molecular weight of about 23 kD as assessed by SDS-PAGE under reducing conditions and includes a domain encoded by the human nucleotide sequence that is defined by eight cysteine residues that are conserved in human vascular endothelial growth factor (VEGF), human platelet derived growth factor A (PDGF-A), and human platelet derived growth factor B (PDGF-B),

wherein said polypeptide lacks any domain that has one or more cysteine motifs of a Balbiani ring 3 protein (BR3P), and

wherein said polypeptide binds to the extracellular domain of human Flt4 receptor tyrosine kinase.

23. A host cell according to claim 22 that expresses a naturally occurring human Flt4 ligand polypeptide encoded by said polynucleotide.

24. A host cell according to claim 22 wherein said polynucleotide is an expression vector, said expression vector including an expression control sequence operatively linked to sequence that encodes said polypeptide.

25. A host cell transformed or transfected with a polynucleotide comprising a nucleotide sequence that encodes the amino acid sequence shown in SEQ ID NO: 33, wherein said host cell expresses a polypeptide encoded by said polynucleotide, said polypeptide including a contiguous portion of SEQ ID NO: 33 that is sufficient to bind to the extracellular domain of human Flt4 receptor tyrosine kinase (Flt4EC),

wherein said contiguous portion includes eight cysteine residues that are conserved in human vascular endothelial growth factor (VEGF), human platelet derived growth factor A (PDGF-A), and human platelet derived growth factor B (PDGF-B),

wherein said polypeptide lacks any portion of SEQ ID NO: 33 that precedes position 1 and lacks any portion

of SEQ ID NO: 33 that has one or more cysteine motifs of a Balbiani ring 3 protein (BR3P), and

wherein said polypeptide has a molecular weight of about 23 kD as assessed by SDS PAGE under reducing conditions and binds to Flt4EC.

26. A host cell according to claim 25 wherein said nucleotide sequence comprises nucleotides 37 to 1086 of the sequence shown in SEQ ID NO: 32.

27. A host cell according to claim 25 wherein said polynucleotide is a vector comprising an expression control sequence operatively linked to the nucleotide sequence that encodes the amino acid sequence shown in SEQ ID NO: 33.

28. A eukaryotic host cell according to claim 22 or 25 that secretes said polypeptide.

29. A host cell comprising the insert of plasmid pFLT4-L, deposited as ATCC accession No. 97231, wherein said host cell expresses and secretes a polypeptide encoded by said insert,

wherein said secreted polypeptide has a molecular weight of about 23 kD as assessed by SDS-PAGE under reducing conditions and binds to human Flt4 receptor tyrosine kinase and includes a domain defined by eight cysteine residues that are conserved in human vascular endothelial growth factor (VEGF), human platelet derived growth factor A (PDGF-A), and human platelet derived growth factor B (PDGF-B).

30. A host cell transformed or transfected with a polynucleotide comprising a nucleotide sequence that encodes a polypeptide that binds to the extracellular domain of human Flt4 receptor tyrosine kinase,

wherein said polynucleotide includes a strand containing a human nucleotide sequence that hybridizes to a DNA comprising the non-coding strand complementary to SEQ ID NO: 32, under the following hybridization conditions:

- (a) hybridization at 42° C. for 20 hours in a solution containing 50% formamide, 5×SSPE, 5×Denhardt's solution, 0.1% SDS and 0.1 mg/ml denatured salmon sperm DNA; and
- (b) washing the filter twice for thirty minutes at room temperature and twice for thirty minutes at 65° C. with a wash solution containing 1×SSC, and 0.1% SDS; and

wherein said host cell expresses and secretes a polypeptide encoded by said polynucleotide, and

wherein said expressed and secreted polypeptide binds the extracellular domain of human Flt4 receptor tyrosine kinase and has a molecular weight of about 23 kD as assessed by SDS-PAGE under reducing conditions.

31. A method for producing a polypeptide that binds the extracellular domain of human Flt4 receptor tyrosine kinase, comprising the steps of:

growing a host cell according to any one of claims 21-27, 29, or 30 under conditions which permit expression by said host cell of said polypeptide; and

isolating said polypeptide from the host cell or the growth medium of the host cell.

32. A method for producing a polypeptide that binds to the extracellular domain (EC) of human Flt4 receptor tyrosine kinase (Flt4), comprising steps of:

growing a host cell comprising a polynucleotide that comprises a nucleotide sequence that encodes the amino acid sequence set forth in SEQ ID NO:33, under conditions in which the host cell expresses and secretes a polypeptide encoded by the polynucleotide; and isolating a polypeptide that binds Flt4 EC from the growth medium of the host cell, said polypeptide having a

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molecular weight of approximately 23 kD as assessed by SDS-PAGE under reducing conditions and having an amino acid sequence comprising a portion of SEQ ID NO:33 effective to bind Flt4 EC.

33. A method according to claim 32 wherein said polynucleotide comprises an expression vector that comprises a nucleotide sequence that encodes the amino acid set forth in SEQ ID NO:33.

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34. A method according to claim 32 wherein said host cell comprises a PC-3 prostatic adenocarcinoma cell (ATCC CRL1435).

35. A method according to claim 32 wherein said polynucleotide comprises the insert of plasmid pFLT4-L, deposited as ATCC Accession No. 97231.

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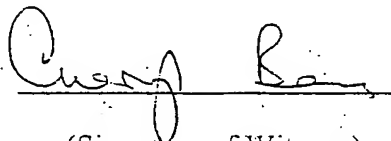
IN THE MATTER OF Australian Patent  
Application Serial No. 696764 by Human  
Genome Sciences, Inc.

-and-

IN THE MATTER OF Opposition thereto by  
Ludwig Institute for Cancer Research

THIS IS Exhibit PAWR-12  
referred to in the Statutory Declaration  
of Peter Adrian Walton Rogers  
made before me

DATED this 12<sup>th</sup> Day of November, 2001

A handwritten signature in cursive script, appearing to read "C. R.", written over a horizontal line.

(Signature of Witness)

Medical Practitioner

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(54) Title: VASCULAR ENDOTHELIAL GROWTH FACTOR 2				
(57) Abstract				
<p>Disclosed is a human VEGF2 polypeptide and DNA (RNA) encoding such VEGF2 polypeptides. Also provided is a procedure for producing such polypeptide by recombinant techniques and antibodies and antagonist against such polypeptide. Such polypeptides may be combined with a suitable pharmaceutical carrier or diluent to provide diagnostic, therapeutic and/or prophylactic effects against various diseases. Also provided are methods of using the antibodies and antagonists to inhibit the action of VEGF2 for therapeutic purposes.</p>				



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### Vascular Endothelial Growth Factor 2

This invention relates to newly identified polynucleotides, polypeptides encoded by such polynucleotides, the use of such polynucleotides and polypeptides, as well as the production of such polynucleotides and polypeptides. More particularly, the polypeptide of the present invention is a human vascular endothelial growth factor 2 (VEGF2). The invention also relates to inhibiting the action of such polypeptide.

The formation of new blood vessels, or angiogenesis, is essential for embryonic development, subsequent growth, and tissue repair. Angiogenesis is an essential part of the growth of human solid cancer, and abnormal angiogenesis is associated with other diseases such as rheumatoid arthritis, psoriasis, and diabetic retinopathy (Folkman, J. and Klagsbrun, M., Science 235:442-447, (1987)).

Several factors are involved in angiogenesis. Both acidic and basic fibroblast growth factor molecules that are mitogens for endothelial cells and other cell types. Angiotropin and angiogenin can induce angiogenesis, although their functions are unclear (Folkman, J., 1993, Cancer Medicine pp. 153-170, Lea and Febiger Press). A highly selective mitogen for vascular endothelial cells is vascular endothelial growth factor or VEGF (Ferrara, N., et al.,

Endocr. Rev. 13:19-32, (1992)). Vascular endothelial growth factor is a secreted angiogenic mitogen whose target cell specificity appears to be restricted to vascular endothelial cells. The murine VEGF gene has been characterized and its expression pattern in embryogenesis has been analyzed. A persistent expression of VEGF was observed in epithelial cells adjacent to fenestrated endothelium, e.g., in choroid plexus and in kidney glomeruli. The data was consistent with a role of VEGF as a multifunctional regulator of endothelial cell growth and differentiation. Breier, G. et al. Development, 114:521-532 (1992).

VEGF can promote angiogenesis. VEGF shares sequence homology with human platelet-derived growth factor, PDGF $\alpha$  and PDGF $\beta$  ( Leung, D.W., et al., Science, 1306-1309, (1989)). The extent of homology is about 21 % and 24 % respectively. Eight cysteine residues are conserved between all three members. Although they are similar, there are specific differences between VEGF and PDGF. While PDGF is a major growth factor for connective tissue, VEGF is highly specific for endothelial cells. VEGF is also known as vascular permeability factor (VPM) and follicle stellate-derived growth factor. It is a heparin-binding dimeric polypeptide.

VEGF has four different forms of 121, 165, 189 and 206 amino acids due to alternative splicing. VEGF121 and VEGF165 are soluble and are capable of promoting angiogenesis, whereas VEGF189 and VEGF206 are bound to heparin containing proteoglycans in the cell surface. The temporal and spatial expression of VEGF has been correlated with physiological proliferation of the blood vessels (Gajdusek, C.M., and Carbon, S.J., Cell Physiol., 139:570-579, (1989)); McNeil, P.L., Muthukrishnan, L., Warder, E., D'Amore, P.A., J. Cell. Biol., 109:811-822, (1989)). Its high affinity binding sites are localized only on endothelial cells in tissue sections (Jakeman, L.B., et al., Clin. Invest. 89:244-253, (1989)).

The factor can be isolated from pituitary cells and several tumor cell lines, and has been implicated in some human gliomas (Plate, K.H. Nature 359:845-848, (1992)). Interestingly, expression of VEGF121 or VEGF165 confers on Chinese hamster ovary cells the ability to form tumors in nude mice (Ferrara, N., et al., J. Clin. Invest. 91:160-170, (1993)). Finally, the inhibition of VEGF function by anti-VEGF monoclonal antibodies was shown to inhibit tumor growth in immune-deficient mice (Kim, K.J., Nature 362:841-844, (1993)).

Vascular permeability factor, also known as VEGF, has also been found to be responsible for persistent microvascular hyperpermeability to plasma proteins even after the cessation of injury, which is a characteristic feature of normal wound healing. This suggests that VPF (or VEGF) is an important factor in wound healing. Brown, L.F. et al., J. Exp. Med., 176:1375-9 (1992).

U.S. Patent No. 5,073,492, issued December 17, 1991 to Chen et al., discloses a method for synergistically enhancing endothelial cell growth in an appropriate environment which comprises adding to the environment, VEGF, effectors and serum-derived factor. Also, vascular endothelial cell growth factor C sub-unit DNA has been prepared by polymerase chain reaction techniques. The DNA encodes a protein that may exist as either a heterodimer or homodimer. The protein is a mammalian vascular endothelial cell mitogen and, as such, is useful for the promotion of vascular development and repair, as disclosed in European Patent Application No. 92302750.2, published September 30, 1992.

In accordance with one aspect of the present invention, there is provided a novel mature polypeptide which is a VEGF2 as well as fragments, analogs and derivatives thereof. The VEGF2 of the present invention is of human origin.

In accordance with another aspect of the present invention, there are provided polynucleotides (DNA or RNA) which encode such polypeptides.

In accordance with still another aspect of the present invention, there is provided a process for producing such polypeptide by recombinant techniques.

In accordance with yet a further aspect of the present invention, there is provided a process for utilizing such polypeptide, or polynucleotide encoding such polypeptide, for therapeutic purposes, for example, as a wound-healing agent, to promote growth of damaged bone and tissue and promote endothelialization as well as for diagnosis of tumors, cancer therapy and to identify and isolate unknown receptors of VEGF2.

In accordance with yet another aspect of the present invention, there is provided an antibody against the VEGF2 and a process for producing such antibody.

In accordance with yet another aspect of the present invention, there are provided antagonist/inhibitors to VEGF2, which may be used to inhibit the action of such polypeptide, for example, to prevent tumor angiogenesis.

These and other aspects of the present invention should be apparent to those skilled in the art from the teachings herein.

The following drawings are illustrative of embodiments of the invention and are not meant to limit the scope of the invention as encompassed by the claims.

Fig. 1 depicts the polynucleotide sequence which encodes for VEGF2, and the corresponding deduced amino acid sequence of the full length VEGF2 polypeptide comprising 350 amino acid residues of which approximately the first 24 amino acids represent the leader sequence. The standard three-letter abbreviation has been used to depict the amino acid sequence.

Fig. 2 shows the homology between growth factor PDGF $\alpha$ , PDGF $\beta$ , VEGF and VEGF2 at the amino acid level.

Fig. 3 shows, in table-form, the percent homology between PDGF $\alpha$ , PDGF $\beta$ , VEGF and VEGF2.

Fig. 4 shows the presence of mRNA for VEGF2 in breast tumor cell lines.

Fig. 5 depicts the results of a Northern blot analysis of VEGF2 in human adult tissues.

Fig. 6 shows the results of running VEGF2 and SDS-PAGE gel after in vitro transcription/translation. The full length and partial VEGF2 cDNA were transcribed and translated in a coupled reaction in the presence of <sup>35</sup>S-methionine. The translated products were analyzed by 4-20% gradient SDS PAGE and exposed to X-ray film.

In accordance with one aspect of the present invention, there is provided an isolated nucleic acid (polynucleotide) which encodes for the mature polypeptide having the deduced amino acid sequence of Figure 1 or for the mature polypeptide encoded by the cDNA of the clone deposited as ATCC Deposit No. \_\_\_\_\_ on \_\_\_\_\_.

A polynucleotide encoding a polypeptide of the present invention may be obtained from early stage human embryo (week 8 to 9) osteoclastomas, adult heart or several breast cancer cell lines. The polynucleotide of this invention was discovered in a cDNA library derived from early stage human embryo week 9. It is structurally related to the VEGF/PDGF family. It contains an open reading frame encoding a protein of about 350 amino acid residues of which approximately the first 24 amino acid residues are likely to be leader sequence such that the mature protein comprises 326 amino acids, and which protein exhibits the highest homology to vascular endothelial growth factor (30% identity), followed by PDGF $\alpha$  (23%) and PDGF $\beta$  (22%), (see Figure 3). It is particularly important that all eight cysteines are conserved within all four members of the family (see boxed areas of Figure 2). In addition, the signature for the PDGF/VEGF family, PXC VXXRXCGCCN, is conserved in VEGF2 (see Figure 2). The

homology between VEGF2, VEGF and the two PDGFs is at the protein sequence level. No nucleotide sequence homology can be detected, and therefore, it would be difficult to isolate the VEGF2 through simple approaches such as low stringency hybridization.

The polynucleotide of the present invention may be in the form of RNA or in the form of DNA, which DNA includes cDNA, genomic DNA, and synthetic DNA. The DNA may be double-stranded or single-stranded, and if single stranded may be the coding strand or non-coding (anti-sense) strand. The coding sequence which encodes the mature polypeptide may be identical to the coding sequence shown in Figure 1 or that of the deposited clone or may be a different coding sequence which coding sequence, as a result of the redundancy or degeneracy of the genetic code, encodes the same, mature polypeptide as the DNA of Figure 1 or the deposited cDNA.

The polynucleotide which encodes for the mature polypeptide of Figure 1 or for the mature polypeptide encoded by the deposited cDNA may include: only the coding sequence for the mature polypeptide; the coding sequence for the mature polypeptide and additional coding sequence such as a leader or secretory sequence or a proprotein sequence; the coding sequence for the mature polypeptide (and optionally additional coding sequence) and non-coding sequence, such as introns or non-coding sequence 5' and/or 3' of the coding sequence for the mature polypeptide.

Thus, the term "polynucleotide encoding a polypeptide" encompasses a polynucleotide which includes only coding sequence for the polypeptide as well as a polynucleotide which includes additional coding and/or non-coding sequence.

The present invention further relates to variants of the hereinabove described polynucleotides which encode for fragments, analogs and derivatives of the polypeptide having the deduced amino acid sequence of Figure 1 or the polypeptide encoded by the cDNA of the deposited clone. The

variant of the polynucleotide may be a naturally occurring allelic variant of the polynucleotide or a non-naturally occurring variant of the polynucleotide.

Thus, the present invention includes polynucleotides encoding the same mature polypeptide as shown in Figure 1 or the same mature polypeptide encoded by the cDNA of the deposited clone as well as variants of such polynucleotides which variants encode for an fragment, derivative or analog of the polypeptide of Figure 1 or the polypeptide encoded by the cDNA of the deposited clone. Such nucleotide variants include deletion variants, substitution variants and addition or insertion variants.

As hereinabove indicated, the polynucleotide may have a coding sequence which is a naturally occurring allelic variant of the coding sequence shown in Figure 1 or of the coding sequence of the deposited clone. As known in the art, an allelic variant is an alternate form of a polynucleotide sequence which have a substitution, deletion or addition of one or more nucleotides, which does not substantially alter the function of the encoded polypeptide.

The present invention also includes polynucleotides, wherein the coding sequence for the mature polypeptide may be fused in the same reading frame to a polynucleotide which aids in expression and secretion of a polypeptide from a host cell, for example, a leader sequence which functions as a secretory sequence for controlling transport of a polypeptide from the cell. The polypeptide having a leader sequence is a preprotein and may have the leader sequence cleaved by the host cell to form the mature form of the polypeptide. The polynucleotides may also encode for a proprotein which is the mature protein plus additional 5' amino acid residues. A mature protein having a prosequence is a proprotein and is an inactive form of the protein. Once the prosequence is cleaved an active mature protein remains.



Thus, for example, the polynucleotide of the present invention may encode for a mature protein, or for a protein having a prosequence or for a protein having both a prosequence and presequence (leader sequence).

The polynucleotides of the present invention may also have the coding sequence fused in frame to a marker sequence which allows for purification of the polypeptide of the present invention. The marker sequence may be a hexahistidine tag supplied by a pQE-9 vector to provide for purification of the mature polypeptide fused to the marker in the case of a bacterial host, or, for example, the marker sequence may be a hemagglutinin (HA) tag when a mammalian host, e.g. COS-7 cells, is used. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein (Wilson, I., et al., Cell, 37:767 (1984)).

The present invention further relates to polynucleotides which hybridize to the hereinabove-described sequences if there is at least 50% and preferably 70% identity between the sequences. The present invention particularly relates to polynucleotides which hybridize under stringent conditions to the hereinabove-described polynucleotides. As herein used, the term "stringent conditions" means hybridization will occur only if there is at least 95% and preferably at least 97% identity between the sequences. The polynucleotides which hybridize to the hereinabove described polynucleotides in a preferred embodiment encode polypeptides which retain substantially the same biological function or activity as the mature polypeptide encoded by the cDNA of Figure 1 or the deposited cDNA.

The deposit(s) referred to herein will be maintained under the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the purposes of Patent Procedure. These deposits are provided merely as a convenience and are not an admission that a deposit is

required under 35 U.S.C. § 112. The sequence of the polynucleotides contained in the deposited materials, as well as the amino acid sequence of the polypeptides encoded thereby, are incorporated herein by reference and are controlling in the event of any conflict with the description of sequences herein. A license may be required to make, use or sell the deposited materials, and no such license is hereby granted.

The present invention further relates to a VEGF2 polypeptide which has the deduced amino acid sequence of Figure 1 or which has the amino acid sequence encoded by the deposited cDNA, as well as fragments, analogs and derivatives of such polypeptide.

The terms "fragment," "derivative" and "analog" when referring to the polypeptide of Figure 1 or that encoded by the deposited cDNA, means a polypeptide which retains essentially the same biological function or activity as such polypeptide. Thus, an analog includes a proprotein which can be activated by cleavage of the proprotein portion to produce an active mature polypeptide.

The polypeptide of the present invention may be a recombinant polypeptide, a natural polypeptide or a synthetic polypeptide, preferably a recombinant polypeptide.

The fragment, derivative or analog of the polypeptide of Figure 1 or that encoded by the deposited cDNA may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or (iv) one in which the additional amino acids are fused to the mature

polypeptide, such as a leader or secretory sequence or a sequence which is employed for purification of the mature polypeptide or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

The polypeptides and polynucleotides of the present invention are preferably provided in an isolated form, and preferably are purified to homogeneity.

The term "isolated" means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or DNA or polypeptide, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotide could be part of a vector and/or such polynucleotide or polypeptide could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

The present invention also relates to vectors which include polynucleotides of the present invention, host cells which are genetically engineered with vectors of the invention and the production of polypeptides of the invention by recombinant techniques.

Host cells are genetically engineered (transduced or transformed or transfected) with the vectors of this invention which may be, for example, a cloning vector or an expression vector. The vector may be, for example, in the form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying the VEGF2 genes. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell

selected for expression, and will be apparent to the ordinarily skilled artisan.

The polynucleotide of the present invention may be employed for producing a polypeptide by recombinant techniques. Thus, for example, the polynucleotide sequence may be included in any one of a variety of expression vehicles, in particular vectors or plasmids for expressing a polypeptide. Such vectors include chromosomal, nonchromosomal and synthetic DNA sequences, e.g., derivatives of SV40; bacterial plasmids; phage DNA; yeast plasmids; vectors derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. However, any other plasmid or vector may be used as long as it is replicable and viable in the host.

As hereinabove described, the appropriate DNA sequence may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease sites by procedures known in the art. Such procedures and others are deemed to be within the scope of those skilled in the art.

The DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s) (promoter) to direct mRNA synthesis. As representative examples of such promoters, there may be mentioned: LTR or SV40 promoter, the E. coli. lac or trp, the phage lambda P<sub>L</sub> promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. The expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression.

In addition, the expression vectors preferably contain a gene to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or

neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in E. coli.

The vector containing the appropriate DNA sequence as herein above described, as well as an appropriate promoter or control sequence, may be employed to transform an appropriate host to permit the host to express the protein. As representative examples of appropriate hosts, there may be mentioned: bacterial cells, such as E. coli, Salmonella typhimurium Streptomyces; fungal cells, such as yeast; insect cells, such as Drosophila and Sf9; animal cells such as CHO, COS or Bowes melanoma; plant cells, etc. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

More particularly, the present invention also includes recombinant constructs comprising one or more of the sequences as broadly described above. The constructs comprise a vector, such as a plasmid or viral vector, into which a sequence of the invention has been inserted, in a forward or reverse orientation. In a preferred aspect of this embodiment, the construct further comprises regulatory sequences, including, for example, a promoter, operably linked to the sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available. The following vectors are provided by way of example. Bacterial: pQE70, pQE-9 (Qiagen), pBs, phagescript, PsiX174, pBluescript SK, pBsKS, pNH8a, pNH16a, pNH18a, pNH46a (Stratagene); pTrc99A, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia). Eukaryotic: pWLneo, pSV2cat, pOG44, pXT1, pSG (Stratagene) pSVK3, pBPV, pMSG, pSVL (Pharmacia). However, any other plasmid or vector may be used as long as they are replicable and viable in the host.

Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) vectors or other vectors with selectable markers. Two appropriate vectors are pKK232-8 and pCM7. Particular named bacterial promoters

include *lacI*, *lacZ*, T3, T7, *gpt*, *lambda P<sub>R</sub>*, *P<sub>L</sub>* and *trp*. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art.

In a further embodiment, the present invention relates to host cells containing the above-described construct. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation (Davis, L., Digner, M., Battey, I., Basic Methods in Molecular Biology, 1986)).

The constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence. Alternatively, the polypeptides of the invention can be synthetically produced by conventional peptide synthesizers.

Mature proteins can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook, et al., Molecular Cloning: A Laboratory Manual, Second Edition, (Cold Spring Harbor, N.Y., 1989), the disclosure of which is hereby incorporated by reference.

Transcription of a DNA encoding the polypeptides of the present invention by higher eukaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300

bp, that act on a promoter to increase its transcription. Examples include the SV40 enhancer on the late side of the replication origin (bp 100 to 270), a cytomegalovirus early promoter enhancer, a polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, e.g., the ampicillin resistance gene of *E. coli* and *S. cerevisiae* TRP1 gene, and a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK),  $\alpha$ -factor, acid phosphatase, or heat shock proteins, among others. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein into the periplasmic space or extracellular medium. Optionally, the heterologous sequence can encode a fusion protein including an N-terminal identification peptide imparting desired characteristics, e.g., stabilization or simplified purification of expressed recombinant product.

Useful expression vectors for bacterial use are constructed by inserting a structural DNA sequence encoding a desired protein together with suitable translation initiation and termination signals in operable reading phase with a functional promoter. The vector will comprise one or more phenotypic selectable markers and an origin of replication to ensure maintenance of the vector and to, if desirable, provide amplification within the host. Suitable prokaryotic hosts for transformation include *E. coli*, *Bacillus subtilis*, *Salmonella typhimurium* and various species within the genera *Pseudomonas*, *Streptomyces*, and

Staphylococcus, although others may also be employed as a matter of choice.

As a representative but nonlimiting example, useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and GEM1 (Promega Biotec, Madison, WI, USA). These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed.

Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter is derepressed by appropriate means (e.g., temperature shift or chemical induction) and cells are cultured for an additional period.

Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification.

Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents.

Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described by Gluzman, Cell, 23:175 (1981), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed



sequences. DNA sequences derived from the SV40 viral genome, for example, SV40 origin, early promoter, enhancer, splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements.

VEGF2 is recovered and purified from recombinant cell cultures by methods used heretofore, including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxyapatite chromatography and lectin chromatography. It is preferred to have low concentrations (approximately 0.1-5mM) of calcium ion present during purification (Price, et al., J. Biol. Chem., 244:917 (1969)). Protein refolding steps can be used, as necessary, in completing configuration of the mature protein. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps.

The polypeptides of the present invention may be a naturally purified product, or a product of chemical synthetic procedures, or produced by recombinant techniques from a prokaryotic or eukaryotic host (for example, by bacterial, yeast, higher plant, insect and mammalian cells in culture). Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated with mammalian or other eukaryotic carbohydrates or may be non-glycosylated.

VEGF2 is useful as a wound healing agent, particularly where it is necessary to re-vascularize damaged tissues, or where new capillary angiogenesis is important. Therefore, it may be used for treatment of full-thickness wounds such as dermal ulcers, including pressure sores, venous ulcers, and diabetic ulcers. In addition, it can be used in the treatment of full-thickness burns and injuries where angiogenesis is desired to prepare the burn in injured sites for a skin graft and flap. In this case, it should be

applied directly at the sites. Similar, VEGF2 can be used in plastic surgery when reconstruction is required following a burn, other trauma, or even for cosmetic purposes.

VEGF2 may also be used to induce the growth of damaged bone, periodontium or ligament tissue. It may be used in periodontal disease where VEGF2 is applied in a methylcellulose gel to the roots of the diseased teeth, the treatment could lead to the formation of new bone and cementum with collagen fiber ingrowths. It can be used for regenerating supporting tissues of teeth, including alveolar bone, cementum and periodontal ligament, that have been damaged by disease and trauma.

Since angiogenesis is important in keeping wounds clean and non-infected, VEGF2 may be used in association with surgery and following the repair of cuts. It should be particularly useful in the treatment of abdominal wounds where there is a high risk of infection.

VEGF2 can be used for the promotion of endothelialization in vascular graft surgery. In the case of vascular grafts using either transplanted or synthetic material, VEGF2 can be applied to the surface of the graft or at the junction to promote the growth of the vascular endothelial cells. One derivation of this is that VEGF2 can be used to repair the damage of myocardial infarction and other occasions where coronary bypass surgery is needed by stimulating the growth of the transplanted tissue. Related to this is the use of VEGF2 to repair the cardiac vascular system after ischemia.

The identification of VEGF2 can be used for the generation of certain inhibitors of vascular endothelial growth factor. Since angiogenesis and neovascularization are essential steps in solid tumor growth, inhibition of angiogenic activity of the vascular endothelial growth factor is very useful to prevent the further growth, retard, or even regress solid tumors. Although the level of expression of

VEGF2 is extremely low in normal tissues including breast, it can be found expressed at moderate levels in at least two breast tumor cell lines that are derived from malignant tumors. It is, therefore, possible that VEGF2 is involved in tumor angiogenesis and growth.

VEGF2 can be used for in vitro culturing of vascular endothelial cells, where it can be added to the conditional medium to a concentration from 10 pg/ml to 10 ng/ml.

The polypeptide of the present invention may also be employed in accordance with the present invention by expression of such polypeptide in vivo, which is often referred to as "gene therapy."

Thus, for example, cells such as bone marrow cells may be engineered with a polynucleotide (DNA or RNA) encoding for the polypeptide ex vivo, the engineered cells are then provided to a patient to be treated with the polypeptide. Such methods are well-known in the art. For example, cells may be engineered by procedures known in the art by use of a retroviral particle containing RNA encoding for the polypeptide of the present invention.

Similarly, cells may be engineered in vivo for expression of the polypeptide in vivo, for example, by procedures known in the art. As known in the art, a producer cell for producing a retroviral particle containing RNA encoding the polypeptide of the present invention may be administered to a patient for engineering cells in vivo and expression of the polypeptide in vivo. These and other methods for administering a polypeptide of the present invention by such methods should be apparent to those skilled in the art from the teachings of the present invention. For example, the expression vehicle for engineering cells may be other than a retroviral particle, for example, an adenovirus, which may be used to engineering cells in vivo after combination with a suitable delivery vehicle.

The polypeptide of the present invention may be employed in combination with a suitable pharmaceutical carrier. Such compositions comprise a therapeutically effective amount of the protein, and a pharmaceutically acceptable carrier or excipient. Such a carrier includes but is not limited to saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The formulation should suit the mode of administration.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. In addition, the polypeptide of the present invention may be employed in conjunction with other therapeutic compounds.

The pharmaceutical compositions may be administered in a convenient manner, such as the oral, and intravenous routes, and is preferably administered topically. The amounts and dosage regimens of VEGF2 administered to a subject will depend on a number of factors, such as the mode of administration, the nature of the condition being treated, the body weight of the subject being treated and the judgment of the prescribing physician. Generally speaking, it is given, for example, in therapeutically effective doses of at least about 10  $\mu$ g/kg body weight and, in most cases, it would be administered in an amount not in excess of about 8 mg/kg body weight per day and preferably the dosage is from about 10  $\mu$ g/kg body weight to about 1 mg/kg body weight daily, taking into the account the routes of administration, symptoms, etc.

The sequences of the present invention are also valuable for chromosome identification. The sequence is specifically

targeted to and can hybridize with a particular location on an individual human chromosome. Moreover, there is a current need for identifying particular sites on the chromosome. Few chromosome marking reagents based on actual sequence data (repeat polymorphism's) are presently available for marking chromosomal location. The mapping of DNAs to chromosomes according to the present invention is an important first step in correlating those sequences with genes associated with disease.

Briefly, sequences can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp) from the cDNA. Computer analysis of the cDNA is used to rapidly select primers that do not span more than one exon in the genomic DNA, thus complicating the amplification process. These primers are then used for PCR screening of somatic cell hybrids containing individual human chromosomes. Only those hybrids containing the human gene corresponding to the primer will yield an amplified fragment.

PCR mapping of somatic cell hybrids is a rapid procedure for assigning a particular DNA to a particular chromosome. Using the present invention with the same oligonucleotide primers, sublocalization can be achieved with panels of fragments from specific chromosomes or pools of large genomic clones in an analogous manner. Other mapping strategies that can similarly be used to map to its chromosome include *in situ* hybridization, prescreening with labeled flow-sorted chromosomes and preselection by hybridization to construct chromosome specific-cDNA libraries.

Fluorescence *in situ* hybridization (FISH) of a cDNA clone to a metaphase chromosomal spread can be used to provide a precise chromosomal location in one step. This technique can be used with cDNA as short as 500 or 600 bases; however, clones larger than 2,000 bp have a higher likelihood of binding to a unique chromosomal location with sufficient signal intensity for simple detection. FISH requires use of

the clone from which the EST was derived, and the longer the better. For example, 2,000 bp is good, 4,000 is better, and more than 4,000 is probably not necessary to get good results a reasonable percentage of the time. For a review of this technique, see Verma et al., Human Chromosomes: a Manual of Basic Techniques. Pergamon Press, New York (1988).

Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. (Such data are found, for example, in V. McKusick, Mendelian Inheritance in Man (available on line through Johns Hopkins University Welch Medical Library). The relationship between genes and diseases that have been mapped to the same chromosomal region are then identified through linkage analysis (coinheritance of physically adjacent genes).

Next, it is necessary to determine the differences in the cDNA or genomic sequence between affected and unaffected individuals. If a mutation is observed in some or all of the affected individuals but not in any normal individuals, then the mutation is likely to be the causative agent of the disease.

With current resolution of physical mapping and genetic mapping techniques, a cDNA precisely localized to a chromosomal region associated with the disease could be one of between 50 and 500 potential causative genes. (This assumes 1 megabase mapping resolution and one gene per 20 kb).

Comparison of affected and unaffected individuals generally involves first looking for structural alterations in the chromosomes, such as deletions or translocations that are visible from chromosome spreads or detectable using PCR based on that cDNA sequence. Ultimately, complete sequencing of genes from several individuals is required to confirm the presence of a mutation and to distinguish mutations from polymorphisms.

The present invention is further directed to inhibiting VEGF2 *in vivo* by the use of antisense technology. Antisense technology can be used to control gene expression through triple-helix formation or antisense DNA or RNA, both of which methods are based on binding of a polynucleotide to DNA or RNA. For example, the 5' coding portion of the mature polynucleotide sequence, which encodes for the polypeptide of the present invention, is used to design an antisense RNA oligonucleotide of from 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription (triple helix - see Lee et al, Nucl. Acids Res., 6:3073 (1979); Cooney et al, Science, 241:456 (1988); and Dervan et al, Science, 251: 1360 (1991), thereby preventing transcription and the production of VEGF2. The antisense RNA oligonucleotide hybridizes to the mRNA *in vivo* and blocks translation of an mRNA molecule into the VEGF2 (antisense - Okano, J. Neurochem., 56:560 (1991); Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL (1988)).

Alternatively, the oligonucleotides described above can be delivered to cells by procedures in the art such that the anti-sense RNA or DNA may be expressed *in vivo* to inhibit production of VEGF2 in the manner described above.

Antisense constructs to VEGF2, therefore, may inhibit the angiogenic activity of the VEGF2 and prevent the further growth or even regress solid tumors, since angiogenesis and neovascularization are essential steps in solid tumor growth. These antisense constructs may also be used to treat rheumatoid arthritis, psoriasis and diabetic retinopathy which are all characterized by abnormal angiogenesis.

The polypeptides, their fragments or other derivatives, or analogs thereof, or cells expressing them can be used as an immunogen to produce antibodies thereto. These antibodies can be, for example, polyclonal or monoclonal antibodies. The present invention also includes chimeric, single chain,

and humanized antibodies, as well as Fab fragments, or the product of an Fab expression library. Various procedures known in the art may be used for the production of such antibodies and fragments..

Antibodies generated against the polypeptide corresponding to a sequence of the present invention can be obtained by direct injection of the polypeptide into an animal or by administering the polypeptide to an animal, preferably a nonhuman. The antibody so obtained will then bind the polypeptide itself. In this manner, even a sequence encoding only a fragment of the polypeptide can be used to generate antibodies binding the whole native polypeptide. Such antibodies can then be used to isolate the polypeptide from tissue expressing that polypeptide. For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique (Kohler and Milstein, 1975, Nature, 256:495-497), the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole, et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96).

Techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce single chain antibodies to immunogenic polypeptide products of this invention.

Neutralization antibodies can be identified and applied to mask the vascular endothelial growth factor, and that has been shown in mice model systems against VEGF. VEGF2 can also be inactivated by certain dominant negative mutants within the gene itself. It is known that both PDGF $\alpha$  and  $\beta$  form either heterodimers or homodimers, and VEGF forms homodimers. Similar interaction between VEGF2 could be expected. These antibodies therefore may be used to block



the angiogenic activity of VEGF2 and retard the growth of solid tumors. These antibodies may also be used to treat inflammation caused by the increased vascular permeability which results from the presence of VEGF2.

These antibodies may further be used in an immunoassay to detect the presence of tumors in certain individuals. Enzyme immunoassay can be performed from the blood sample of an individual. Elevated levels of VEGF2 can be considered diagnostic of cancer.

The present invention is also directed to antagonist/inhibitors of the polypeptides of the present invention. The antagonist/inhibitors are those which inhibit or eliminate the function of the polypeptide.

Thus, for example, antagonists bind to a polypeptide of the present invention and inhibit or eliminate its function. The antagonist, for example, could be an antibody against the polypeptide which binds to the polypeptide or, in some cases, an oligonucleotide. An example of an inhibitor is a small molecule which binds to and occupies the catalytic site of the polypeptide thereby making the catalytic site inaccessible to substrate such that normal biological activity is prevented. Examples of small molecules include but are not limited to small peptides or peptide-like molecules.

Truncated versions of VEGF2 can also be produced that are capable of interacting with wild type VEGF2 form dimers that fail activate endothelial cell growth, therefore inactivated the endogenous VEGF2. Or, mutant forms of VEGF2 form dimers themselves and occupies the ligand binding domain of the proper tyrosine Kinase receptors on the target cell surface, but fail to activate the cell growth.

Alternatively, antagonists to the polypeptides of the present invention may be employed which bind to the receptors to which a polypeptide of the present invention normally binds. The antagonists may be closely related proteins such

that they recognize and bind to the receptor sites of the natural protein, however, they are inactive forms of the natural protein and thereby prevent the action of VEGF2 since receptor sites are occupied. In these ways, the action of the VEGF2 is prevented and the antagonist/inhibitors may be used therapeutically as an anti-tumor drug by occupying the receptor sites of tumors which are recognized by VEGF2 or by inactivating VEGF2 itself. The antagonist/inhibitors may also be used to prevent inflammation due to the increased vascular permeability action of VEGF2. The antagonist/inhibitors may also be used to treat solid tumor growth, diabetic retinopathy, psoriasis and rheumatoid arthritis.

The antagonist/inhibitors may be employed in a composition with a pharmaceutically acceptable carrier, e.g., as hereinabove described.

The present invention will be further described with reference to the following examples; however, it is to be understood that the present invention is not limited to such examples. All parts or amounts, unless otherwise specified, are by weight.

In order to facilitate understanding of the following examples, certain frequently occurring methods and/or terms will be described.

"Plasmids" are designated by a lower case p preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are either commercially available, publicly available on an unrestricted basis, or can be constructed from available plasmids in accord with published procedures. In addition, equivalent plasmids to those described are known in the art and will be apparent to the ordinarily skilled artisan.

"Digestion" of DNA refers to catalytic cleavage of the DNA with a restriction enzyme that acts only at certain sequences in the DNA. The various restriction enzymes used

herein are commercially available and their reaction conditions, cofactors and other requirements were used as would be known to the ordinarily skilled artisan. For analytical purposes, typically 1  $\mu$ g of plasmid or DNA fragment is used with about 2 units of enzyme in about 20  $\mu$ l of buffer solution. For the purpose of isolating DNA fragments for plasmid construction, typically 5 to 50  $\mu$ g of DNA are digested with 20 to 250 units of enzyme in a larger volume. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation times of about 1 hour at 37°C are ordinarily used, but may vary in accordance with the supplier's instructions. After digestion the reaction is electrophoresed directly on a polyacrylamide gel to isolate the desired fragment.

Size separation of the cleaved fragments is performed using 8 percent polyacrylamide gel described by Goeddel, D. et al., *Nucleic Acids Res.*, 8:4057 (1980).

"Oligonucleotides" refers to either a single stranded polydeoxynucleotide or two complementary polydeoxynucleotide strands which may be chemically synthesized. Such synthetic oligonucleotides have no 5' phosphate and thus will not ligate to another oligonucleotide without adding a phosphate with an ATP in the presence of a kinase. A synthetic oligonucleotide will ligate to a fragment that has not been dephosphorylated.

"Ligation" refers to the process of forming phosphodiester bonds between two double stranded nucleic acid fragments (Maniatis, T., et al., *Id.*, p. 146). Unless otherwise provided, ligation may be accomplished using known buffers and conditions with 10 units of T4 DNA ligase ("ligase") per 0.5  $\mu$ g of approximately equimolar amounts of the DNA fragments to be ligated.

Unless otherwise stated, transformation was performed as described by the method of Graham, F. and Van der Eb, A., Virology, 52:456-457 (1973).

#### Example 1

##### Expression pattern of VEGF2 in human tissues and breast cancer cell lines

Northern blot analysis was carried out to examine the levels of expression of VEGF2 in human tissues and breast cancer cell lines in human tissues. Total cellular RNA samples were isolated with RNazol™ B system (Biotecx Laboratories, Inc.). About 10 µg of total RNA isolated from each breast tissue and cell line specified was separated on 1% agarose gel and blotted onto a nylon filter, (Molecular Cloning, Sambrook Fritsch, and Maniatis, Cold Spring Harbor Press, 1989). The labeling reaction was done according to the Stratagene Prime-It kit with 50 ng DNA fragment. The labeled DNA was purified with a Select-G-50 column from 5' Prime -- 3 Prime, Inc. The filter was then hybridized with radioactive labeled full length VEGF2 gene at 1,000,000 cpm/ml in 0.5 M NaPO<sub>4</sub> and 7 % SDS overnight at 65°C. After wash twice at room temperature and twice at 60°C with 0.5 X SSC, 0.1 % SDS, the filters were then exposed at -70°C overnight with intensifying screen. A message of 1.6 Kd was observed in 2 breast cancer cell lines. Lane #4 represents a very tumorigenic cell line that is estrogen independent for growth. See Figure 4. Also, 10 µg of total RNA from 10 human adult tissues were separated on an agarose gel and blotted onto a nylon filter. The filter was then hybridized with radioactively labeled VEGF2 probe in 7% SDS, 0.5 M NaPO<sub>4</sub>, pH 7.2; 1% BSA overnight at 65°C. Following wash in 0.2 X SSC at 65°C, the filter was exposed to film for 24 days at -70°C with intensifying screen. See Figure 5.

#### Example 2

Expression of VEGF2 by in vitro transcription and translation

The VEGF2 cDNA was transcribed and translated in vitro to determine the size of the translatable polypeptide encoded by the full length and partial VEGF2 cDNA. The full length and partial cDNA inserts of VEGF2 in the pBluescript SK vector were amplified by PCR with three pairs of primers, 1) M13-reverse and forward primers; 2) M13-reverse primer and VEGF primer F4; 3) M13-reverse primer and VEGF primer F5. The sequence of these primers are as follows.

M13-2 reverse primer:

5'-ATGCTTCCGGCTCGTATG-3'

This sequence is located upstream of the 5' end of the VEGF2 cDNA insert in the pBluescript vector and is in an anti-sense orientation as the cDNA. A T3 promoter sequence is located between this primer and the VEGF2 cDNA.

M13-2 forward primer:

5'GGGTTTTCCCAGTCACGAC-3'

This sequence is located downstream of the 3' end of the VEGF2 cDNA insert in the pBluescript vector and is in an anti-sense orientation as the cDNA insert.

VEGF primer F4:

5'-CCACATGGTTCAGGAAAGACA-3'

This sequence is located within the VEGF2 cDNA in an anti-sense orientation from bp 1259-1239, which is about 169 bp away from the 3' end of the stop codon and about 266 bp before the last nucleotide of the cDNA.

PCR reaction with all three pairs of primers produce amplified products with T3 promoter sequence in front of the cDNA insert. The first and third pairs of primers produce PCR products that encode the full polypeptide of VEGF2. The second pair of primers produce PCR product that misses 36 amino acids coding sequence at the C-terminus of the VEGF2 polypeptide.

Approximately 0.5 ug of PCR product from first pair of primers, 1 ug from second pair of primers, 1 ug from third

pair of primers were used for in vitro transcription/translation. The in vitro transcription/translation reaction was performed in a 25 ul of volume, using the T<sub>N</sub>T™ Coupled Reticulocyte Lysate Systems (promega, CAT# L4950). Specifically, the reaction contains 12.5 ul of T<sub>N</sub>T rabbit reticulocyte lysate 2 ul of T<sub>N</sub>T reaction buffer, 1 ul of T3 polymerase, 1 ul of 1 mM amino acid mixtrue (minus methionine), 4 ul of <sup>35</sup>S-methionine (>1000 Ci/mmol, 10 mCi/ml), 1 ul of 40 U/ul; RNasin ribonuclease inhibitor, 0.5 or 1 ug of PCR products. Nuclease-free H<sub>2</sub>O were added to bring the me to 25 ul. The reaction was incubated at 30°C for 2 hours. Five microliters of the reaction product was analyzed on a 4-20% gradient SDS-PAGE gel. After fixing in 25% isopropanol and 10% acetic acid, the gel was dried and exposed to an X-ray film overnight at 70°C.

As shown in Fig. 6, PCR products containing the full length VEGF2 cDNA and the cDNA missing 266 bp in the 3' untranslated region (3'-UTR) produced the same length of translated products, whose molecular weights are estimated to be 38-40 dk (lanes 1 & 3). The cDNA missing all the 3'UTR and missing sequence encoding the C-terminal 36 amino acids was translated into a polypeptide with an estimated molecular weight of 36-38 kd (lane 2).

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: HU, ET AL.
- (ii) TITLE OF INVENTION: Vascular Endothelial Growth  
Factor 2
- (iii) NUMBER OF SEQUENCES: 2
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: CARELLA, BYRNE, BAIN, GILFILLAN,  
CECCHI, STEWART & OLSTEIN
  - (B) STREET: 6 BECKER FARM ROAD
  - (C) CITY: ROSELAND
  - (D) STATE: NEW JERSEY
  - (E) COUNTRY: USA
  - (F) ZIP: 07068
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: 3.5 INCH DISKETTE
  - (B) COMPUTER: IBM PS/2
  - (C) OPERATING SYSTEM: MS-DOS
  - (D) SOFTWARE: WORD PERFECT 5.1
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER: 08/207,550
  - (B) FILING DATE: 8 MARCH 1994
  - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE;

## (viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: FERRARO, GREGORY D.  
(B) REGISTRATION NUMBER: 36,134  
(C) REFERENCE/DOCKET NUMBER: 325800-148

## (ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: 201-994-1700  
(B) TELEFAX: 201-994-1744

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 1525 BASE PAIRS  
(B) TYPE: NUCLEIC ACID  
(C) STRANDEDNESS: SINGLE  
(D) TOPOLOGY: LINEAR

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CGAGGCCACG	GCTTATGCAA	GCAAAGATCT	GGAGGAGCAG	TTACGGTCTG	TGTCCAGTGT	60
AGATGAACTC	ATGACTGTAC	TCTACCCAGA	ATATTGGAAG	ATGTACAAGT	GTCAGCTAAG	120
GAAAGGAGGC	TGGCAACATA	ACAGAGAACA	GGCCAACTC	AACTCAAGGA	CAGAAGAGAC	180
TATAAAATTT	CGTGCAGCAC	ATTATAATAC	AGAGATCTTG	AAAAGTATTG	ATAATGAGTG	240
GAGAAAGACT	CAATGCATGC	CACGGGAGGT	GTGTATAGAT	GTGGGGAAGG	AGTTTGGAGT	300
CGCGACAAAC	ACCTTCTTTA	AACCTCCATG	TGTGTCCGTC	TACAGATGTA	GGGGTTGCTG	360
CAATAGTGAG	GGGCTGCAGT	GCAATGAACAC	CAGCAGGAGC	TACCTCAGCA	AGACGTTATT	420
TGAAATTACA	GTGCCTCTCT	CTCAAGGCCG	CAAACCACTA	ACAATCAGTT	TTGCCAATCA	480
CACTTCCTGC	CGATGCATGT	CTAACTGGA	TGTTTACAGA	CAAGTTCATT	CCATTATTAG	540
ACGTTCCCTG	CCAGCAACAC	TACCACAGTG	TCAGGCAGCG	AACAAGACCT	GCCCCACCAA	600
TTACATGTGG	AATAATCACA	TCTGCAGATG	CCTGGCTCAG	GAAGATTTTA	TGTTTTCTC	660
CGATGCTGGA	GATGACTCAA	CAGATGGATT	CCATGACATC	TGTGGACCAA	ACAAGGAGCT	720
GGATGAAGAC	ACCTGTCACT	GTGTCTGCAG	AGCGGGGCTT	CGGCCTGCCA	GCTGTGGACC	780
CCACAAAGAA	CTAGACAGAA	ACTCATGCCA	GTGTGTCTGT	AAAAACAAAC	TCTTCCCCAG	840
CCAATGRGGG	GCCAACCGAC	AATTTGATGA	AAACACATGC	CAGTGTGTAT	GTAAGAAGAC	900
CTGCCCCAGA	AATCAACCCC	TAAATCCTGG	AAAATGTGCC	TGTGAATGTA	CAGAAAGTCC	960



ACAGAAATGC	TTGTTAAAAG	GAAAGAAGTT	CCACCACCAA	ACATGCAGCT	GTTACAGACG	1020
GCCATGTACC	AACCGCCAGA	AGGCTTGTA	GCCAGGATTT	TCATATAGTG	AAGAAGTGTG	1080
TCGTTGTGTC	CCTTCATATT	GGCAAAGACC	ACAAATGAGC	TAAGATTGTA	CTGTTTTCCA	1140
GTTTCATCGAT	TTTCTATTAT	GGAAAACTGT	GTTGCCACAG	TAGAACTGTC	TGTGAACAGA	1200
GAGACCCCTTG	TGGGTCCATG	CTAACAAAGA	CAAAAGTCTG	TCTTTCCTGA	ACCATGTGGA	1260
TAACTTTACA	GAAATGGACT	GGAGCTCATC	TGCAAAAGGC	CTCTTGTAAG	GACTGGTTTT	1320
CTGCCAATGA	CCAAACAGCC	AAGATTTTCC	TCTTGTGATT	TCTTTAAAAG	AATGACTATA	1380
TAATTTATTT	CCACTAAAAA	TATTGTTTCT	GCATTCATTT	TTATAGCAAC	AACAATTGGT	1440
AAAACCTCACT	GTGATCAATA	TTTTTATATC	ATGCAAAATA	TGTTTAAAAT	AAAATGAAAA	1500
TTGTATTATA	AAAAAAAAAA	AAAAA				1525

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 350 AMINO ACIDS  
 (B) TYPE: AMINO ACID  
 (C) STRANDEDNESS:  
 (D) TOPOLOGY: LINEAR

## (ii) MOLECULE TYPE: PROTEIN

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met	Thr	Val	Lys	Tyr	Pro	Glu	Tyr	Trp	Lys	Met	Tyr	Lys	Cys	Gln
			-20						-15				-10	
Leu	Arg	Lys	Gly	Gly	Trp	Gln	His	Asn	Arg	Glu	Gln	Ala	Asn	Leu
			-5						1				5	
Asn	Ser	Arg	Thr	Glu	Glu	Thr	Ile	Lys	Phe	Ala	Ala	Ala	His	Tyr
			10						15				20	
Asn	Thr	Glu	Ile	Leu	Lys	Ser	Ile	Asp	Asn	Glu	Trp	Arg	Lys	Thr
			25						30				35	
Gln	Cys	Met	Pro	Arg	Glu	Val	Cys	Ile	Asp	Val	Gly	Lys	Glu	Phe
			40						45				50	
Gly	Val	Ala	Thr	Asn	Thr	Phe	Phe	Lys	Pro	Pro	Cys	Val	Ser	Val
			55						60				65	
Tyr	Arg	Cys	Gly	Gly	Cys	Cys	Asn	Ser	Glu	Gly	Leu	Gln	Cys	Met
			70						75				80	

Asn	Thr	Ser	Thr	Ser	Tyr	Leu	Ser	Lys	Thr	Leu	Phe	Glu	Ile	Thr	
				85					90					95	
Val	Pro	Leu	Ser	Gln	Gly	Pro	Lys	Pro	Val	Thr	Ile	Ser	Phe	Ala	
				100					105					110	
Asn	His	Thr	Ser	Cys	Arg	Cys	Met	Ser	Lys	Leu	Asp	Val	Tyr	Arg	
				115					120					125	
Gln	Val	His	Ser	Ile	Ile	Arg	Arg	Ser	Leu	Pro	Ala	Thr	Leu	Pro	
				130					135					140	
Gln	Cys	Gln	Ala	Ala	Asn	Lys	Thr	Cys	Pro	Thr	Asn	Tyr	Met	Trp	
				145					150					155	
Asn	Asn	His	Ile	Cys	Arg	Cys	Leu	Ala	Gln	Glu	Asp	Phe	Met	Phe	
				160					165					170	
Ser	Ser	Asp	Ala	Gly	Asp	Asp	Ser	Thr	Asp	Gly	Phe	His	Asp	Ile	
				175					180					185	
Cys	Gly	Pro	Asn	Lys	Glu	Leu	Asp	Glu	Glu	Thr	Cys	Gln	Cys	Val	
				190					195					200	
Cys	Arg	Ala	Gly	Leu	Arg	Pro	Ala	Ser	Cys	Gly	Pro	His	Lys	Glu	
				205					210					215	
Leu	Asp	Arg	Asn	Ser	Cys	Gln	Cys	Val	Cys	Lys	Asn	Lys	Leu	Phe	
				220					225					230	
Pro	Ser	Gln	Cys	Gly	Ala	Asn	Arg	Glu	Phe	Asp	Glu	Asn	Thr	Cys	
				235					240					245	
Gln	Cys	Val	Cys	Lys	Arg	Thr	Cys	Pro	Arg	Asn	Gln	Pro	Leu	Asn	
				250					255					260	
Pro	Gly	Lys	Cys	Ala	Cys	Glu	Cys	Thr	Glu	Ser	Pro	Gln	Lys	Cys	
				265					270					275	
Cys	Leu	Leu	Lys	Gly	Lys	Lys	Phe	His	His	Gln	Thr	Cys	Ser	Cys	
				280					285					290	
Tyr	Arg	Arg	Pro	Cys	Thr	Asn	Arg	Gln	Lys	Ala	Cys	Glu	Pro	Gly	
				295					300					305	
Phe	Ser	Tyr	Ser	Glu	Glu	Val	Cys	Arg	Cys	Val	Pro	Ser	Tyr	Trp	
				310					315					320	
Gln	Arg	Pro	Gln	Met	Ser										
				325											

## WHAT IS CLAIMED IS:

1. An isolated polynucleotide encoding for VEGF2, said polynucleotide selected from the group consisting of polynucleotide encoding for the VEGF2 polypeptide having the deduced amino acid sequence of Figure 1 or an active fragment, analogue or derivative of said polypeptide; polynucleotide encoding for the VEGF2 polypeptide having the amino acid sequence encoded by the cDNA contained in ATCC Deposit No. 75698 or an active fragment analogue or derivative of said polypeptide.
2. The polynucleotide of Claim 1 wherein the polynucleotide is DNA.
3. The polynucleotide of Claim 1 wherein the polynucleotide is RNA.
4. The polynucleotide of Claim 1 wherein the polynucleotide is genomic DNA.
5. The polynucleotide of Claim 2 wherein said polynucleotide encodes for VEGF2 having the deduced amino acid sequence of Figure 1.
6. The polynucleotide of Claim 2 wherein said polynucleotide encodes for the VEGF2 polypeptide encoded by the cDNA of ATCC Deposit No. 75698.
7. The polynucleotide of Claim 1 having the coding sequence for VEGF2 as shown in Figure 1.
8. The polynucleotide of Claim 2 having the coding sequence for VEGF2 deposited as ATCC Deposit No. 75698.
9. A vector containing the DNA of Claim 2.
10. A host cell genetically engineered with the vector of Claim 9.
11. A process for producing a polypeptide comprising: expressing from the host cell of Claim 10 the polypeptide encoded by said DNA.
12. A process for producing cells capable of expressing a polypeptide comprising genetically engineering cells with the vector of Claim 9.

13. An isolated DNA hybridizable to the DNA of Claim 2 and encoding a polypeptide having VEGF2 activity.
14. A polypeptide selected from the group consisting of (i) a VEGF2 polypeptide having the deduced amino acid sequence of Figure 1 and active fragments, analogs and derivatives thereof and (ii) a VEGF2 polypeptide encoded by the cDNA of ATCC Deposit No. 75698 and active fragments, analogs and derivatives of said polypeptide.
15. The polypeptide of Claim 14 wherein the polypeptide is VEGF2 having the deduced amino acid sequence of Figure 1.
16. An antibody against the polypeptide of claim 14.
17. An antagonist against the polypeptide of claim 14.
18. A method for the treatment of a patient having need of VEGF2 comprising: administering to the patient a therapeutically effective amount of the polypeptide of claim 14.
19. A method for the treatment of a patient having need to inhibit VEGF2 comprising: administering to the patient a therapeutically effective amount of an antagonist against the polypeptide of Claim 14.
20. A pharmaceutical composition comprising the polypeptide of Claim 14 and a pharmaceutically acceptable carrier.
21. The method of Claim 18 wherein said therapeutically effective amount of the polypeptide is administered by providing to the patient DNA encoding said polypeptide and expressing said polypeptide *in vivo*.

## FIG. 1A

1 CGAGGCCACGGCTTATGCAAGCAAGATCTGGAGGAGCAGGTACGGTCTGTGTCAGTGT  
71 AGATGAACCTCAGTGTACTCTACCCAGAAATATTGGAAAATGTACAAGTGTCTAGCTAAG  
M T V L Y P E Y W K M Y K C Q L R  
121 GAAAGGAGGCTGGCAACNTAACAGAGAACAGGCCCACTCAACTCAAGGACAGAAAGAGAC  
K G G W Q H N R E Q A N L N S R T E E T  
181 TATAAAATTGTGCAGCACATTATAATACAGAGATCTTGAAAAGTATGTATAATGAGTG  
I K F A A A H Y N T E I L K S I D N E W  
241 GAGAAAGACTCAATGCTGCCACGGGAGGTGTGTATAGATGTGGGAAGGAGTTGGAGT  
R K T Q C M P R E V C I D V G K E F G V  
301 CGCGACAAACACCTTCTTTAAACCTCCATGTGTGTCGGTCTACAGATGTGGGGGTTGCTG  
A T N T F F K P P C V S V Y R C G C C  
361 CAATAGTGAGGGGCTGCAGTGCATGAAACACCAGCAGGCTACCTCAGCAAGACGTTATT  
N S E G L Q C M N T S T S Y L S K T L F  
421 TGAATTACAGTGCCTCTCTCTCAAGGCCCAACCAAGTAACAATCAGTTTGGCCATCA  
MATCH WITH FIG. 1B

## FIG. 1B

MATCH WITH FIG. 1A

```
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
E I T V P L S Q G P K P V T I S F A N H
481 CACTTCCGCGATGCATGTCCTAACTGGATGTTACAGACAAGTTCATTCCTATTAG
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
T S C R C M S K L D V Y R Q V H S I I R
541 ACGTTCCCTGCCAGCAACACTACCACAGTGTCCAGCGCAGCAAGACCTGCCCCCAACAA
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
R S L P A T L P Q C Q A A N K T C P T N
601 TTACATGTGGAATAATCACATCTGCAGATGCCCTGGCTCAGGAAGATTTTATGTTTCCTC
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
Y M W N N H I C R C L A Q E D F M F S S
661 GGATGCTGGAGATGACTCAACAGATGGATTCCATGCATCTGTGGACCAACAAGGAGCT
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
D A G D D S T D G F H D I C G P N K E L
721 GGATCAAGAGACCTGTCAGTGTCTGTGCAGAGCGGGGCTTCGGCCCTGCCAGCTGTGGACC
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
D E E T C Q C V C R A G L R P A S C G P
781 CCACAAAGAACTAGACAGAAACTCATGCCAGTGTGTCTGTAAAAACAACCTCTCCCCAG
-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
H K E L D R N S C Q C V C K N K L F P S
841 CCAATGTGGGGCCAAACCGAGAATTGTATGAAACACATGCCAGTGTGTATGTAAAGAAC
```

MATCH WITH FIG. 1C

## MATCH WITH FIG. 1B FIG. 1C

```

-----+-----+-----+-----+-----+
Q C G A N R E F D E N T C Q C V C K R T
201 CTGCCCCAGAAATCAACCCCTAAATCCTCGAAATGTGCCCTGTGAATGTACAGAAAGTCC
-----+-----+-----+-----+-----+
C P R N Q P L N P G K C A C E C T E S P
961 ACAGAAATGCTTGTAAAGGAAAGAGTTCCACCACCAACATGCAGCTGTACAGACG
-----+-----+-----+-----+-----+
Q K C L L K G K K F H H Q T C S C Y R R
1021 GCCATGTACGAACCCGCCAGAAGGCTTGTGAGCCAGGATTTTCATATAGTGAAGAAGTGTG
-----+-----+-----+-----+-----+
P C T N R Q K A C E P G F S Y S E E V C
1081 TCGTTGTCCCTTCATATTGCAAGACCAACAATGAGCTAAGATGTACTGTTTCCCA
-----+-----+-----+-----+-----+
R C V P S Y W Q R P Q M S
1141 GTTCATCGATTTTCTATATGAAACTGTGTGCCACAGTAGAAGTGTCTGTGAACAGA
-----+-----+-----+-----+-----+
GAGACCCCTGTGGTCCATGCTAACAAAGACAAAGTCTCTTTCCTGAACCATGTGGA
1201
-----+-----+-----+-----+-----+

```

MATCH WITH FIG. 1D

MATCH WITH FIG. 1C

FIG. 1D

```
1261  TAACTTTACAGAAATGGACTGGAGCTCATCTGC AAAAGGCCCTCTTGTAAGACTGGT TTT  
-----+-----+-----+-----+-----+-----+-----+  
1321  CTGCCAATGACCAACAGCCAAGATTTTCCTCTTGTCGATTTC TTTAAAGAAATGACTATA  
-----+-----+-----+-----+-----+-----+-----+  
1381  TAATTTATTTCCACTAAATAATATGTTTCTGCAATTCATTTTATAGCAACAACAATTGGT  
-----+-----+-----+-----+-----+-----+-----+  
1441  AAACTCACTGTGATCAATAATTTTATATCATGC AAAAATATGTTTAAAAATGA AAA  
-----+-----+-----+-----+-----+-----+-----+  
1501  TTGTATTATAAAAAA AAAAAA  
-----+-----+-----+-----+-----+-----+-----+
```



## FIG. 2A

50

1

pdgfa .MRTLACLLL LGGCYLAHV L AEEAETPREV IERLARSQIH SIRD LQRLLE  
 pdgfb MNRCWA.LFL SLCCYLRLVS AEGDPIPEEL YEMLS DHSIR SFDDLQRL LH  
 Vegf .....MNFLL SWVHWSLALL LY.....  
 Vegf2 .....MTV LYPEYWKMYK CQ.....

100

51

pdgfa IDSVGSEDSL DTS LRAHG V H ATKHVPEKRP LPIRRKR SI. ....EEAVP  
 pdgfb GDP.GEEDGA ELDLNMTRSH SGGELES... .LARGRRSLG SLTIAEPAMI  
 Vegf APMAE.....GGGQ NHHEVVKFMD .VYQR.....  
 Vegf2 REQANLNSRT EETIKFAAAH YNTEILKSID NEWRK.....

5/9

SUBSTITUTE SHEET (RULE 26)

150

101

pdgfa AVCKTRTVIY EIPRSQVDPT SANFLIWPCC VEVKRC TGCC NTSSVKCQPS  
 pdgfb AECKTRTEVF EISRRLLDRT NANFLVWPCC VEVQRC SGCC NNRNVQCRPT  
 Vegf SYCHPIETLV DIFQEYPDEI ..EYIFKPSC VPLMRCGGCC NDEGLECVPT  
 Vegf2 TQCMPREVCI DVGKEFGVAT ..NTFFKPCC VSVYRCGGCC NSEGLQCMNT

200

151

pdgfa RVHHRSVKVA KVEYVRKKPK LKEVQVRLEE HLETC..... AT.....  
 pdgfb QVQLRPVQVR KIEIVRKKPI FKKATVTLED HLAKC..... ETVAAARPVT  
 Vegf EESNITMQIM RIK.PH..QG QHIGEMSFLQ HNKCECRPKK DRARQEKKS V  
 Vegf2 STSYLSKTLF EIT.VPLSQG PKPVTISFAN HTSCRCMSKL DVYRQVHSII

MATCH WITH FIG. 2B

## FIG. 2B

MATCH WITH FIG. 2A

201	250
pdgfa	.....TSLNPD YREEDTDVR. ....
pdgfb	RSPGGSQEQR AKTPQTRVTI RTVRVRRPPK GKHRKFKKHTH DKTALKETLG
veg	RGK.....GKGQKRKRK KSRYKSWSVY VGARCCCLMPW SLPGPHP....
veg2	RRSLPATLPQ CQAANKTCPT NYMWNHICR CLAQEDFMFS SDAGDDSDTG
251	300
pdgfa	.....
pdgfb	A.....
veg	....CGP... ..CSE RRKHLFVODP QTCKCSCKNT
veg2	FHDICGNKE LDEETQCVC RAGLRPASC GPHKEL...DR NSCQCVCCKNK
301	350
pdgfa	.....
pdgfb	.....
veg	..DSRCKARQ LEINERTC RC DKPRR.....
veg2	LFPSQCGANR .EFDENTCQC VCKRTCPRNQ PLNPGKCACE CTESPOKCLL
351	398
pdgfa	.....
pdgfb	.....
veg	.....
veg2	KGKFFHHQTC SCYRRPCTNR QKACEPGFSY SEEVCRCPVS YWQRPQMS

## FIG. 3

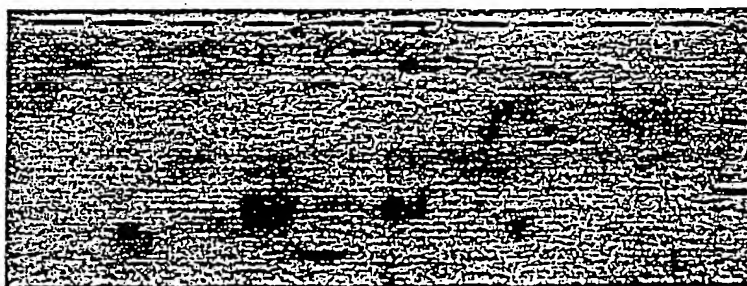
PERCENTAGE (%) OF AMINO ACID IDENTITIES  
BETWEEN EACH PAIR OF GENES IS SHOWN IN THE  
FLOWING TABLE.

	PDGF $\alpha$	PDGF $\beta$	VEGF	VEGF2
PDGF $\alpha$				
PDGF $\beta$	48.0			
VEGF	20.7	22.7		
VEGF2	23.5	22.4	30.0	

## FIG. 4

EXPRESSION OF VEGF2 IN  
IN HUMAN BREAST TUMOR CELL LINES

1 2 3 4 5 6 7 8 9



28S

18S

1. NORMAL BREAST

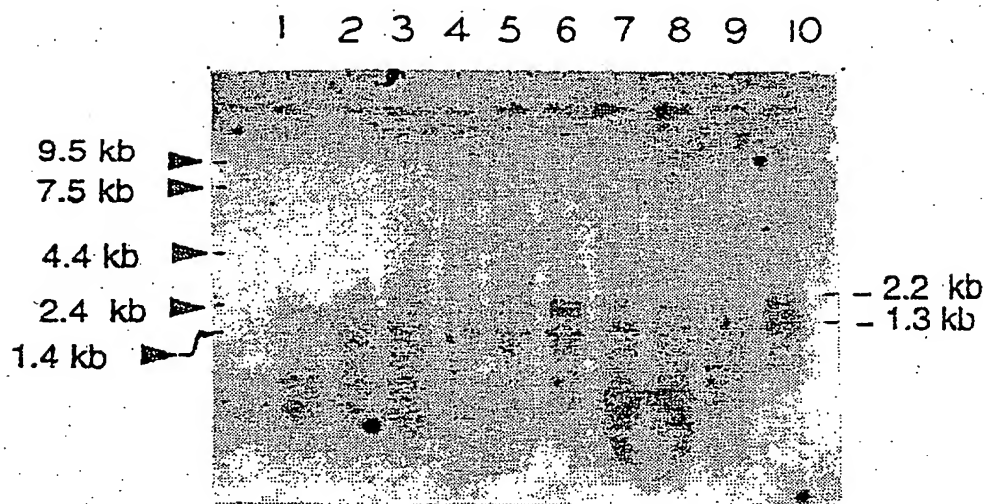
2. BREAST CANCER

3-9. BREAST CANCER CELL LINES

7/9

SUBSTITUTE SHEET (RULE 26)

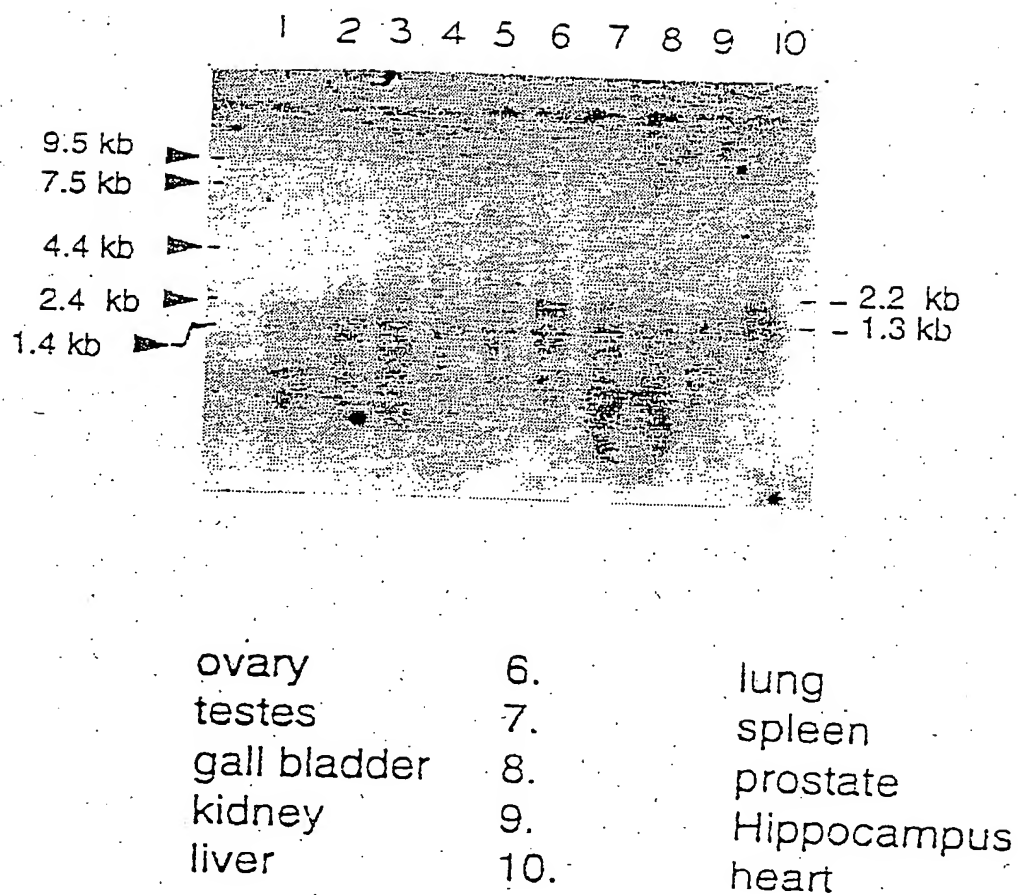
## FIG. 5



- |    |              |     |             |
|----|--------------|-----|-------------|
| 1. | ovary        | 6.  | lung        |
| 2. | testes       | 7.  | spleen      |
| 3. | gall bladder | 8.  | prostate    |
| 4. | kidney       | 9.  | Hippocampus |
| 5. | liver        | 10. | heart       |

Expression of VEGF2 mRNA in human adult tissues.

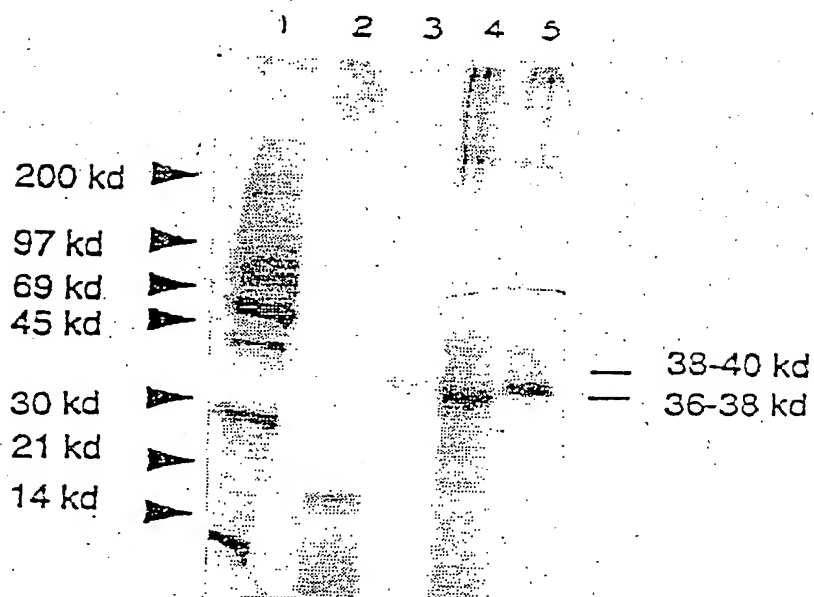
## FIG. 5



Expression of VEGF2 mRNA in human adult tissues.

## FIG. 6

In vitro transcription/translation  
of VEGF2 protein.



- Lane 1: 14-C and rainbow M.W. marker  
 Lane 2: FGF control  
 Lane 3: VEGF2 (M13-reverse & forward primers)  
 Lane 4: VEGF2 (M13-reverse & VEGF-F4 primers)  
 Lane 5: VEGF2 (M13-reverse & VEGF-F5 primers)

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US94/05291

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : C12N 15/00, 15/18; C07K 13/00; A61K 37/36

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 172.3, 240.2, 252.3, 252.33, 320.1, 514/2, 12; 530/350, 399; 536/23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS AND DIALOG (files 5, 155, 351, 357, 358) search terms: VEGF, VEGF2, VEGFII, vascular endothelial growth factor

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP, A, 0,476,983 (BAYNE ET AL) 25 March 1992, see entire document.	1-15
A	JOURNAL OF CELLULAR BIOCHEMISTRY, Volume 47, issued 1991, Ferrara et al, "The Vascular Endothelial Growth Factor Family of Polypeptides", pages 211-218, see entire document.	1-15

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*A*	document defining the general state of the art which is not considered to be of particular relevance	*T*	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*E*	earlier document published on or after the international filing date	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*L*	document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*O*	document referring to an oral disclosure, use, exhibition or other means	*Z*	document member of the same patent family
*P*	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

19 OCTOBER 1994

Date of mailing of the international search report

04 NOV 1994

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

MARIANNE PORTA ADLEN

Telephone No. (703) 308-0196

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US94/05291

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
1-15

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.



# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US94/05291

## A. CLASSIFICATION OF SUBJECT MATTER: US CL :

435/69.1, 172.3, 240.2, 252.3, 252.33, 320.1; 514/2, 12; 530/350, 399; 536/23.5

## BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

Group I. Claims 1-15, drawn to a polynucleotide sequence, vector, host cell, process of producing the host cell, process of producing the protein, and protein, classified in at least Class 536, subclass 23.5, for example.

Group II. Claim 16, drawn to an antibody, classified in at least Class 530, subclass 387.1, for example.

Group III. Claim 17, drawn to an antagonist, classified in at least Class 530, subclass 350, for example.

Group IV. Claims 18-21, drawn to methods of treatment, classified in at least Class 514, subclass 44, for example.

The claims of the four groups are directed to different inventions which are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept. Note that PCT Rule 13 does not provide for multiple products or methods within a single application.

AUSTRALIA

*Patents Act 1990*

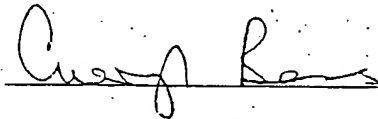
IN THE MATTER OF Australian Patent  
Application Serial No. 696764 by Human  
Genome Sciences, Inc.

-and-

IN THE MATTER OF Opposition thereto, by  
Ludwig Institute for Cancer Research

THIS IS Exhibit PAWR-13  
referred to in the Statutory Declaration  
of Peter Adrian Walton Rogers  
made before me

DATED this 12<sup>th</sup> Day of November, 2001



(Signature of Witness)

Medical Practitioner



US005935820A

United States Patent [19]

Hu et al.

[11] Patent Number: 5,935,820

[45] Date of Patent: Aug. 10, 1999

[54] POLYNUCLEOTIDES ENCODING  
VASCULAR ENDOTHELIAL GROWTH  
FACTOR 2

0506477 9/1992 European Pat. Off.  
9705250 2/1997 WIPO  
9709427 3/1997 WIPO  
9717442 5/1997 WIPO

[75] Inventors: Jing-Shan Hu, Sunnyvale, Calif.;  
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the People's Republic of China

[73] Assignee: Human Genome Science, Inc.,  
Rockville, Md.

[21] Appl. No.: 08/824,996

[22] Filed: Mar. 27, 1997

#### Related U.S. Application Data

[62] Division of application No. 08/207,550, Mar. 8, 1994,  
abandoned.

[51] Int. Cl.<sup>6</sup> ..... C12N 15/18; C12N 15/63;  
C12N 1/21; C12N 5/00

[52] U.S. Cl. .... 435/69.4; 435/70.1; 435/325;  
435/320.1; 435/243; 536/23.4; 536/23.51;  
530/399

[58] Field of Search ..... 435/69.4, 325,  
435/243, 320.1, 70.1; 530/399; 536/23.51,  
23.4

[56] References Cited

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5,073,492 12/1991 Chen et al. .... 435/240.2  
5,219,739 6/1993 Tischer et al. .... 435/69.4  
5,326,695 7/1994 Andersson et al. .... 435/70.1

#### FOREIGN PATENT DOCUMENTS

0476983 3/1992 European Pat. Off.

#### OTHER PUBLICATIONS

Eichmann, A., et al., Development 125(4):743-752 (1998).  
George et al. Macromolecular Seq. and Syn. Selected Meth  
& Applic pp. 127-149, 1998.  
U.S. application No. 08/340011, filed Nov. 1994.  
U.S. application No. 08/510133, filed Aug. 1995.  
U.S. application No. 08/585895, filed Jan. 1996.  
U.S. application No. 08/601132, filed Feb. 1996.  
U.S. application No. 08/671573, filed Jun. 1996.  
U.S. provisional application No. 60/03491, filed Sep. 1995.  
U.S. application No. 08/8554374, filed Nov. 1995.  
Pajusola, K., et al., Cancer Research. 52:5738-5743 (1992).  
Pajusola, K., et al., Oncogene 8:2931-2937 (1993).  
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munications 165(3):1198-1206 (1989).  
Leung, D.W., et al., Science 246:1306-1309 (1989).  
Breier, G., et al., Development 114:521-532 (1992).  
Ferrara, et al., Journal of Cellular Biochemistry 47:211-218  
(1991).

Primary Examiner—John Ulm

Assistant Examiner—Christine Saoud

Attorney, Agent, or Firm—Human Genome Sciences Inc

[57] ABSTRACT

The present invention relates to polynucleotides encoding a novel human protein called Vascular Endothelial Growth Factor 2, or VEGF-2, as well as mature forms, proproteins, and fragments of VEGF-2. Also provided are vectors, host cells, and recombinant methods for producing VEGF-2. The invention further relates to polynucleotides that hybridize to polynucleotides of the present invention.

116 Claims, 10 Drawing Sheets

1 CGAGGCCACGGCTTATGCAAGCAAGATCTGGAGGAGCAGTTACGGTCTGTGTCCAGTGT  
-----+-----+-----+-----+-----+-----+-----+  
71 AGATGAACTCATGACTGTACTCTACCCAGAAATATTGGAATGTACAAGTTCAGCTAAG  
-----+-----+-----+-----+-----+-----+-----+  
M T V L Y P E Y W K M Y K C Q L R  
-----+-----+-----+-----+-----+-----+-----+  
121 GAAAGGAGGCTGGCAACATAACAGAACAGGCCAACCTCAAGTCAAGACAGAAAGAC  
-----+-----+-----+-----+-----+-----+-----+  
K G G W Q H N R E Q A N L N S R T E E T  
-----+-----+-----+-----+-----+-----+-----+  
181 TATAAAATTGCTGCAGCACATTATAACAGAGATCTTGAAAAGTATTGATAATGAGTG  
-----+-----+-----+-----+-----+-----+-----+  
I K F A A A H Y N T E I L K S I D N E W  
-----+-----+-----+-----+-----+-----+-----+  
241 GAGAAAGACTCAATGCATGCCACGGAGGTGTGTATAGATGTGGGAAGGAGTTTGGAGT  
-----+-----+-----+-----+-----+-----+-----+  
R K T Q C M P R E V C I D V G K E F G V  
-----+-----+-----+-----+-----+-----+-----+  
301 CGGACAAACACCTTCTTTAAACCTCCATGTGTGTCCGTCTACAGATGTGGGGGTGCTG  
-----+-----+-----+-----+-----+-----+-----+  
A T N T F F K P P C V S V Y R C G G C C  
-----+-----+-----+-----+-----+-----+-----+

FIG. 1A

361 CAATAGTGAGGGCTGCAGTGCATGAACACGACGAGCTACCTCAGCAAGACGTTATT  
-----+-----+-----+-----+-----+  
N S E G L Q C M N T S T S Y L S K T L F  
-----+-----+-----+-----+-----+  
421 TGAAATTACAGTGCCTCTCTCTCAAGGCCCAACACGTAACAATCAGTTTGTCCCAATCA  
-----+-----+-----+-----+-----+  
E I T V P L S Q G P K P V T I S F A N H  
-----+-----+-----+-----+-----+  
481 CACTTCCTGCCGATGCTCTAAACTGGATGTTACAGACAAGTTCATTCCATTATTAG  
-----+-----+-----+-----+-----+  
T S C R C M S K L D V Y R Q V H S I I R  
-----+-----+-----+-----+-----+  
541 ACGTTCCTGCCAGCAACACTACCACAGTGTCAAGCAGCGAACAAGACCTGCCCCACCAA  
-----+-----+-----+-----+-----+  
R S L P A T L P Q C Q A A N K T C P T N  
-----+-----+-----+-----+-----+  
601 TTACATGTGGAATAATCACATCTGCAGATGCCCTGGCTCAGGAAGATTTATGTTTCCCTC  
-----+-----+-----+-----+-----+  
Y M W N N H I C R C L A Q E D F M F S S  
-----+-----+-----+-----+-----+  
661 GGATGCTGGAGATGACTCAACAGATGGATTCCATGACATCTGTGGACCAACAAGAGCT  
-----+-----+-----+-----+-----+  
D A G D D S T D G F H D I C G P N K E L  
-----+-----+-----+-----+-----+

FIG. 1B

721 GGATGAAGAGACCTGTCAGTGTGTCTGCAGACGGGGCTTCGGCCCTGCCAGCTGTGGACC  
D E E T C Q C V C R A G L R P A S C G P  
781 CCACAAAGAACTAGACAGAAACTCATGCCAGTGTGTCTGTAAAAACAACCTCTTCCCCAG  
H K E L D R N S C Q C V C K N K L F P S  
841 CCAATGTGGGCCCAACCGAGAATTGTATGAACAACACATGCCAGTGTGTATGTAAAGAAC  
Q C G A N R E F D E N T C Q C V C K R T  
901 CTGCCCCAGAAATCAACCCCTAAATCCTGGAAATGTGCCCTGTGAATGTACAGAAAGTCC  
C P R N Q P L N P G K C A C E C T E S P  
961 ACAGAAATGCTTGTAAAGGAAGAAGTTCACCACCACCAACATGCCAGCTGTACAGACG  
Q K C L L K G K K F H H Q T C S C Y R R  
1021 GCCATGTACGAACCGCCAGAAGGCTTGTGAGCCAGGATTTTCATATAGTGAAGAAGTGTG  
P C T N R Q K A C E P G F S Y S E F V C

FIG.1C

FIG. 1D

1 50  
Pdga .MRTLACLL LCCYLAVL AEEAIPREV IERLARSQIH SIRDQLRLE  
Pdgb .MNRCAW.LFL SLCCYLRLVS AEGDPIPEEL YEMLSOHSIR SFDDLQRLH  
Vegf . . . . . MNFL SWHWSLALL LY . . . . . LHHAKWSQA  
Vegf2 . . . . . MTV LTYPEYKMYK CQ . . . . . LRKGGWQHIN

51 100  
Pdga .IDSVGSEDSL .DTSLRAHGVH .ATKHVPEKRP .LPIRRKRSL . . . . . EEAVP  
Pdgb .GDP .GEEDGA .ELDNLMTSRH .SGCELES . . . . . LARGRRSLG .SLTIAEPAMI  
Vegf .APMAE . . . . . GCGQ .NHHEVVKFMD .VYQR . . . . .  
Vegf2 .REQANLNSRT .EETIKFAAAH .YNTILKSID .NEWRK . . . . .

101 150  
Pdga .AVCKTRTVLY .EIPRSOVDPT .SANFLIMPPC .VEVKRCTGCC .NTSSVKQPPS  
Pdgb .AECKTRTEVF .EISRRLLDRT .NANFLVMPPC .VEVQRCGGCC .NNRNVCQRPT  
Vegf .SYCHPIETLV .DIFQEYPDEI .EYIFKPPSC .VPLMRCCGCC .NDEGLECVPT  
Vegf2 .TQCMPREVCI .DVGKEFGVAT .NTFFKPPC .VSVYRCGGCC .NSEGLQCMNT

151 200  
Pdga .RVHHRSVKVA .KVEYVRKKPK .LKEVQVRLEE .HLEQAC . . . . . AT  
Pdgb .QVQLRPVQVR .KIEIVRKKPI .FKKATVTLED .HLACKQ . . . . . ETVAARPVT  
Vegf .EESNITMQIM .RIK .PH .QG .QHIGEMSFLO .HNKCECRPKK .DRARQEKKS  
Vegf2 .STSYLSKTLF .EIT .VPLSQG .PKPVTISFAN .HTSCRCMSKL .DVYRQVHSII

FIG. 2A



201  
Pdgfa .....TSLNPD YREEDIDVR. 250  
Pdgfb RSPGCSQEQ AKTPQTRVTI RTVVRVRPPK GKHKFKHIH DKTALKETLG  
Vegf RGK.....GKGQKRKRK KSRYSWSVY VGARCCCLMPW SLPGPHP  
Vegf2 RRLPATLPQ COAANKTCPT NYMNNHICR CLAQEDFMFS SDAGDDSTDG

251  
Pdgfa ..... 300  
Pdgfb A.....  
Vegf .....CGP  
Vegf2 FHDICGNKE LDEETCCVC RAGLRPASCG PHKEL...DR NSCQCVCCKNK

301  
Pdgfa ..... 350  
Pdgfb .....  
Vegf DSRCKARQ LELNERICRC DKPRR  
Vegf2 LFPSQCCANR EFDENTCQC VCKRICPRNQ PLNPGKACE CTESPOKCLL

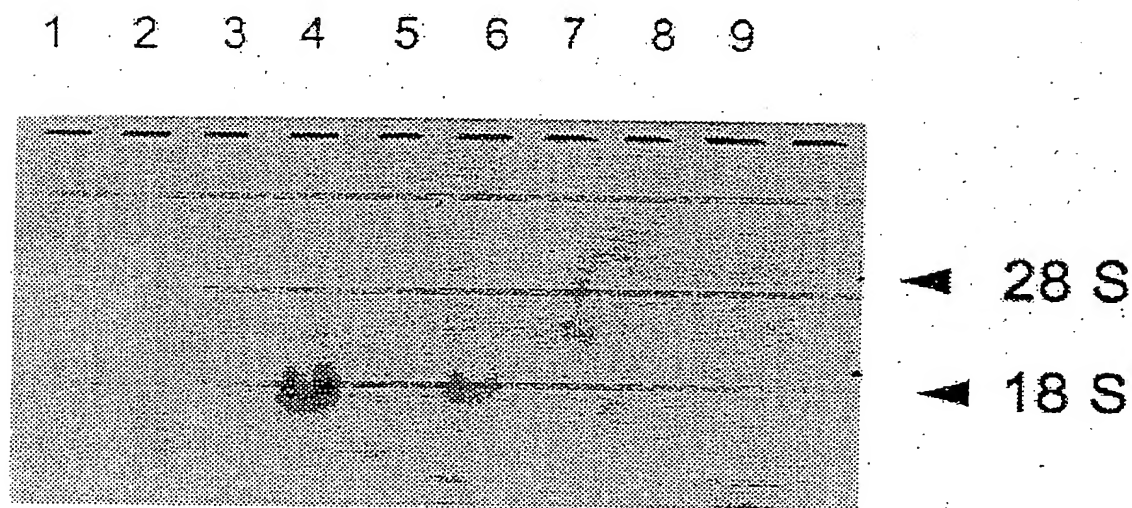
351  
Pdgfa ..... 398  
Pdgfb .....  
Vegf .....  
Vegf2 KGKFFHHQTC SCYRRPCTNR QKACEPGFSY SEEVCRCPVS YWORPQMS

FIG.2B

PERCENTAGE (%) OF AMINO ACID IDENTITIES BETWEEN EACH PAIR OF GENES IS SHOWN IN THE FOLLOWING TABLE				
	PDGF $\alpha$	PDGF $\beta$	VEGF	VEGF2
PDGF $\alpha$				
PDGF $\beta$	48.0			
VEGF	20.7	22.7		
VEGF2	23.5	22.4	30.0	

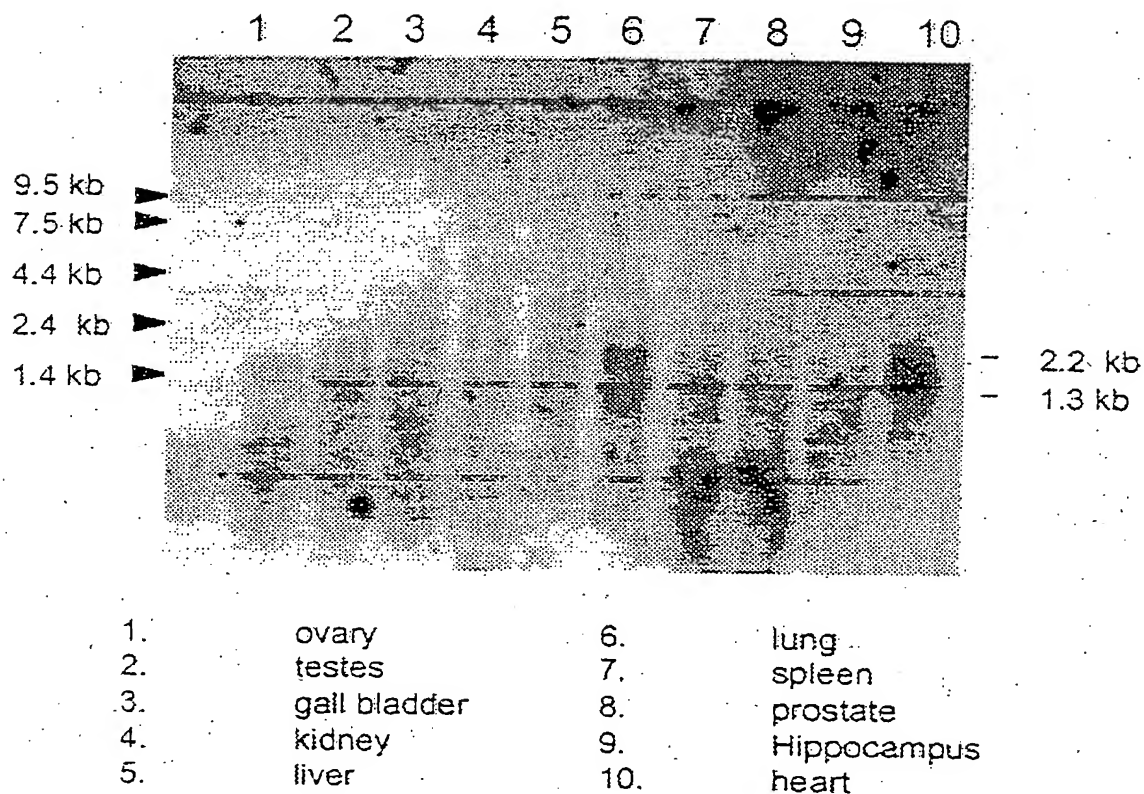
FIG. 3

# Expression of VEGF2 mRNA in Human Breast Tumor Cells



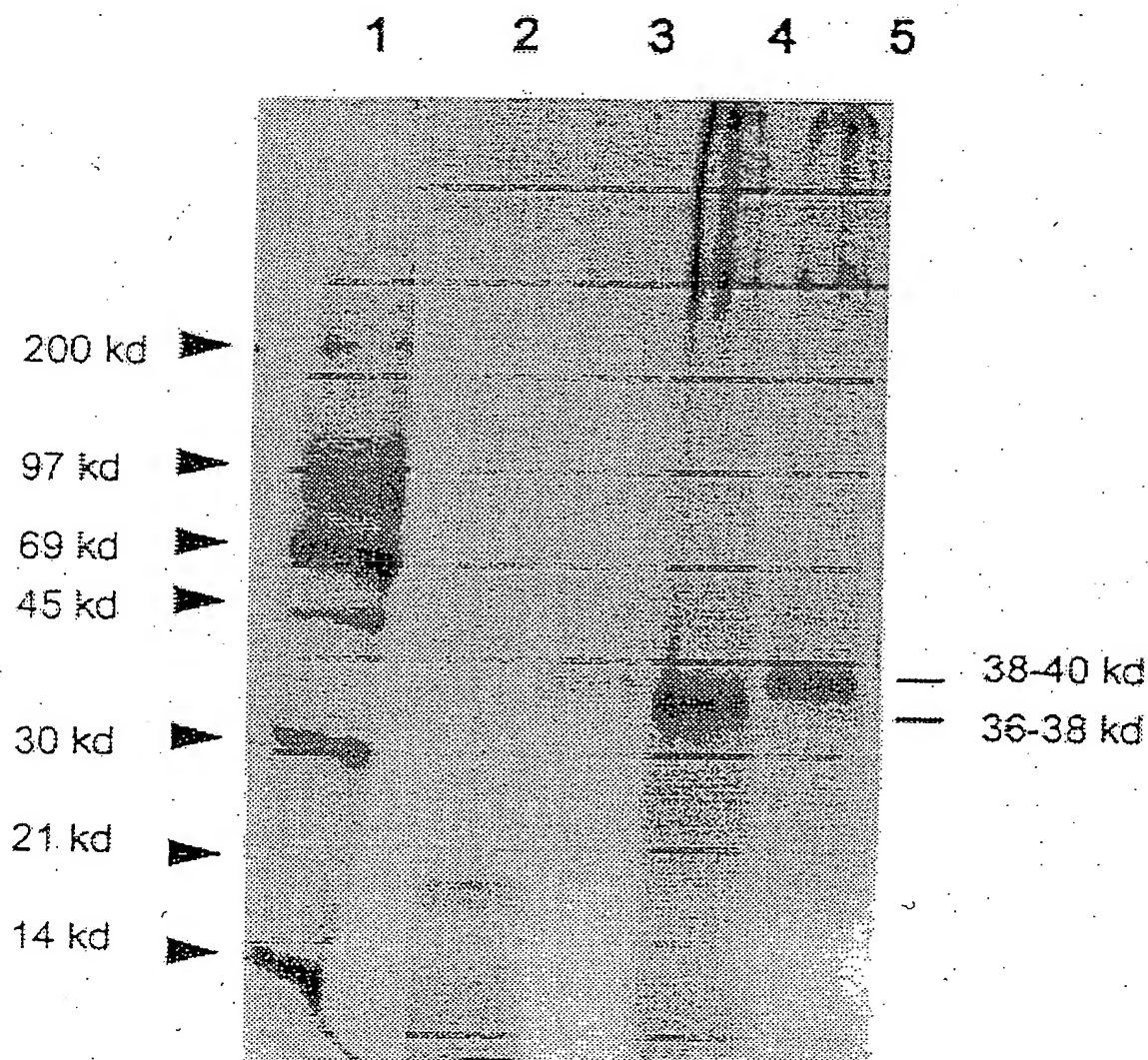
Lane 1. normal breast tissue  
Lane 2. breast tumor tissue  
Lane 3-9. breast tumor cell lines.

FIG.4



Expression of VEGF2 mRNA in human adult tissues.

FIG.5



Lane 1: 14-C and rainbow M.W. marker  
Lane 2: FGF control  
Lane 3: VEGF2 (M13-reverse & forward primers)  
Lane 4: VEGF2 (M13-reverse & VEGF-F4 primers)  
Lane 5: VEGF2 (M13-reverse & VEGF-F5 primers)

FIG. 6

# POLYNUCLEOTIDES ENCODING VASCULAR ENDOTHELIAL GROWTH FACTOR 2

This is a Division of application Ser. No. 08/207,550, filed Mar. 8, 1994, now abandoned.

This invention relates to newly identified polynucleotides, polypeptides encoded by such polynucleotides, the use of such polynucleotides and polypeptides, as well as the production of such polynucleotides and polypeptides. More particularly, the polypeptide of the present invention is a human vascular endothelial growth factor 2 (VEGF2). The invention also relates to inhibiting the action of such polypeptide.

The formation of new blood vessels, or angiogenesis, is essential for embryonic development, subsequent growth, and tissue repair. Angiogenesis is an essential part of the growth of human solid cancer, and abnormal angiogenesis is associated with other diseases such as rheumatoid arthritis, psoriasis, and diabetic retinopathy (Folkman, J. and Klagsbrun, M., *Science* 235:442-447, (1987)).

Several factors are involved in angiogenesis. Both acidic and basic fibroblast growth factor molecules that are mitogens for endothelial cells and other cell types. Angiotropin and angiogenin can induce angiogenesis, although their functions are unclear (Folkman, J., 1993, *Cancer Medicine* pp. 153-170, Lea and Febiger Press). A highly selective mitogen for vascular endothelial cells is vascular endothelial growth factor or VEGF (Ferrara, N., et al., *Endocr. Rev.* 13:19-32, (1992)). Vascular endothelial growth factor is a secreted angiogenic mitogen whose target cell specificity appears to be restricted to vascular endothelial cells. The murine VEGF gene has been characterized and its expression pattern in embryogenesis has been analyzed. A persistent expression of VEGF was observed in epithelial cells adjacent to fenestrated endothelium, e.g., in choroid plexus and in kidney glomeruli. The data was consistent with a role of VEGF as a multifunctional regulator of endothelial cell growth and differentiation. Breier, G. et al. *Development*, 114:521-532 (1992).

VEGF can promote angiogenesis. VEGF shares sequence homology with human platelet-derived growth factor, PDGFA and PDGFB (Leung, D. W., et al., *Science*, 1306-1309, (1989)). The extent of homology is about 21% and 24% respectively. Eight cysteine residues are conserved between all three members. Although they are similar, there are specific differences between VEGF and PDGF. While PDGF is a major growth factor for connective tissue, VEGF is highly specific for endothelial cells. VEGF is also known as vascular permeability factor (VPM) and follicle stellate-derived growth factor. It is a heparin-binding dimeric polypeptide.

VEGF has four different forms of 121, 165, 189 and 206 amino acids due to alternative splicing. VEGF121 and VEGF165 are soluble and are capable of promoting angiogenesis, whereas VEGF189 and VEGF206 are bound to heparin containing proteoglycans in the cell surface. The temporal and spatial expression of VEGF has been correlated with physiological proliferation of the blood vessels (Gajdusek, C. M., and Carbon, S. J., *Cell Physiol.*, 139:570-579, (1989)); McNeil, P. L., Muthukrishnan, L., Warder, E., D'Amore, P. A., *J. Cell. Biol.*, 109:811-822, (1989)). Its high affinity binding sites are localized only on endothelial cells in tissue sections (Jakeman, L. B., et al., *Clin. Invest.* 89:244-253, (1989)). The factor can be isolated from pituitary cells and several tumor cell lines, and has been implicated in some human gliomas (Plate, K. H. *Nature* 359:845-848, (1992)).

Interestingly, expression of VEGF121 or VEGF165 confers on Chinese hamster ovary cells the ability to form tumors in nude mice (Ferrara, N., et al., *J. Clin. Invest.* 91:160-170, (1993)). Finally, the inhibition of VEGF function by anti-VEGF monoclonal antibodies was shown to inhibit tumor growth in immune-deficient mice (Kim, K. J., *Nature* 362:841-844, (1993)).

Vascular permeability factor, also known as VEGF, has also been found to be responsible for persistent microvascular hyperpermeability to plasma proteins even after the cessation of injury, which is a characteristic feature of normal wound healing. This suggests that VPF (or VEGF) is an important factor in wound healing. Brown, L. F. et al., *J. Exp. Med.*, 176:1375-9 (1992).

U.S. Pat. No. 5,073,492, issued Dec. 17, 1991 to Chen et al., discloses a method for synergistically enhancing endothelial cell growth in an appropriate environment which comprises adding to the environment, VEGF, effectors and serum-derived factor. Also, vascular endothelial cell growth factor C sub-unit DNA has been prepared by polymerase chain reaction techniques. The DNA encodes a protein that may exist as either a heterodimer or homodimer. The protein is a mammalian vascular endothelial cell mitogen and, as such, is useful for the promotion of vascular development and repair, as disclosed in European Patent Application No. 92302750.2, published Sept. 30, 1992.

In accordance with one aspect of the present invention, there is provided a novel mature polypeptide which is a VEGF2 as well as fragments, analogs and derivatives thereof. The VEGF2 of the present invention is of human origin.

In accordance with another aspect of the present invention, there are provided polynucleotides (DNA or RNA) which encode such polypeptides. In accordance with still another aspect of the present invention, there is provided a process for producing such polypeptide by recombinant techniques.

In accordance with yet a further aspect of the present invention, there is provided a process for utilizing such polypeptide, or polynucleotide encoding such polypeptide, for therapeutic purposes, for example, as a wound-healing agent, to promote growth of damaged bone and tissue and promote endothelialization as well as for diagnosis of tumors; cancer therapy and to identify and isolate unknown receptors of VEGF2.

In accordance with yet another aspect of the present invention, there is provided an antibody against the VEGF2 and a process for producing such antibody.

In accordance with yet another aspect of the present invention, there are provided antagonist/inhibitors to VEGF2, which may be used to inhibit the action of such polypeptide, for example, to prevent tumor angiogenesis.

These and other aspects of the present invention should be apparent to those skilled in the art from the teachings herein.

## BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings are illustrative of embodiments of the invention and are not meant to limit the scope of the invention as encompassed by the claims.

FIGS. 1A, 1B, 1C and 1D (FIG. 1A shows the first portions of the polynucleotide sequence encoding VEGF2 and the amino acid sequence for VEGF2, and FIGS. 1B, 1C and 1D, respectively continue with the sequential portions of each sequence began in FIG. 1A) collectively depict the polynucleotide sequence (SEQ ID NO:1) which encodes VEGF2, and the corresponding amino acid sequence (SEQ

ID NO:2) for the VEGF2 polypeptide comprising 350 amino acid residues of which approximately the first 24 amino acids represent the leader sequence. The standard one-letter codes are utilized to depict the amino acid residues encoded by the polynucleotide triplets. The polynucleotide sequence (SEQ ID NO:1) which encodes for VEGF2, and the corresponding deduced amino acid sequence (SEQ ID NO:2) of the full length VEGF2 polypeptide comprising 350 amino acid residues of which approximately the first 24 amino acids represent the leader sequence. The standard three-letter abbreviation has been used to depict the amino acid sequence.

FIGS. 2A and 2B collectively depict polypeptide sequences in alignment and show the alignment of VEGF2 with the other growth factor PDGF $\alpha$ , PDGF $\beta$ , and VEGF. FIG. 2A depicts N-terminal portions of the polypeptide sequences and FIG. 2A continues with C-terminal portions of the polypeptide sequences. The four lines in each comparative row depict, respectively, the PDGF $\alpha$  polypeptide sequence (SEQ ID NO:7), the PDGF $\beta$  polypeptide sequence (SEQ ID NO:8), the VEGF polypeptide sequence (SEQ ID NO:9) and the VEGF2 polypeptide sequence. The amino acid residues are illustrated in FIGS. 2A and 2B by the standard one-letter codes.

FIG. 3 shows, in table-form, the percent homology between PDGF $\alpha$ , PDGF $\beta$ , VEGF and VEGF2.

FIG. 4 shows the presence of mRNA for VEGF2 in breast tumor cell lines.

FIG. 5 depicts the results of a Northern blot analysis of VEGF2 in human adult tissues.

FIG. 6 shows the results of running VEGF2 and SDS-PAGE gel after in vitro transcription/translation. The full length and partial VEGF2 cDNA were transcribed and translated in a coupled reaction in the presence of <sup>35</sup>S-methionine. The translated products were analyzed by 4–20% gradient SDS PAGE and exposed to X-ray film.

In accordance with one aspect of the present invention, there is provided an isolated nucleic acid (polynucleotide) which encodes for the mature polypeptide having the deduced amino acid sequence of SEQ ID NO:2 or for the mature polypeptide encoded by the cDNA of the clone deposited as ATCC Deposit No. 75698, on Mar. 4, 1994, with ATCC, 10801 University Boulevard, Manassas, Va. 20110-2209. Since the strain referred to is being maintained under the terms of the Budapest Treaty, it will be made available to a patent office signatory to the Budapest Treaty. If a patent should issue which is directed to the present invention, upon the issuance of such a patent the deposited strain of ATCC 75698 will be irrevocably and without restriction released to the public, excepting for those restrictions permitted by enforcement of the patent.

A polynucleotide encoding a polypeptide of the present invention may be obtained from early stage human embryo (week 8 to 9) osteoclastomas, adult heart or several breast cancer cell lines. The polynucleotide of this invention was discovered in a cDNA library derived from early stage human embryo week 9. It is structurally related to the VEGF/PDGF family. It contains an open reading frame encoding a protein of about 350 amino acid residues of which approximately the first 24 amino acid residues are likely to be leader sequence such that the mature protein comprises 326 amino acids, and which protein exhibits the highest homology to vascular endothelial growth factor (30% identity), followed by PDGF $\alpha$  (23%) and PDGF $\beta$  (22%), (see FIG. 3). It is particularly important that all eight cysteines are conserved within all four members of the

family (see boxed areas of FIG. 2). In addition, the signature for the PDGF/VEGF family, PXCVCXXRCXGCCN, (SEQ ID NO:3) is conserved in VEGF2 (see FIG. 2). The homology between VEGF2, VEGF and the two PDGFs is at the protein sequence level. No nucleotide sequence homology can be detected, and therefore, it would be difficult to isolate the VEGF2 through simple approaches such as low stringency hybridization.

The polynucleotide of the present invention may be in the form of RNA or in the form of DNA, which DNA includes cDNA, genomic DNA, and synthetic DNA. The DNA may be double-stranded or single-stranded, and if single stranded may be the coding strand or non-coding (anti-sense) strand. The coding sequence which encodes the mature polypeptide may be identical to the coding sequence shown in SEQ ID NO:1 or that of the deposited clone or may be a different coding sequence which coding sequence, as a result of the redundancy or degeneracy of the genetic code, encodes the same, mature polypeptide as the DNA of SEQ ID NO:1 or the deposited cDNA.

The polynucleotide which encodes for the mature polypeptide of FIG. 1 or for the mature polypeptide encoded by the deposited cDNA may include: only the coding sequence for the mature polypeptide; the coding sequence for the mature polypeptide and additional coding sequence such as a leader or secretory sequence or a proprotein sequence; the coding sequence for the mature polypeptide (and optionally additional coding sequence) and non-coding sequence, such as introns or non-coding sequence 5' and/or 3' of the coding sequence for the mature polypeptide.

Thus, the term "polynucleotide encoding a polypeptide" encompasses a polynucleotide which includes only coding sequence for the polypeptide as well as a polynucleotide which includes additional coding and/or non-coding sequence.

The present invention further relates to variants of the hereinabove described polynucleotides which encode for fragments, analogs and derivatives of the polypeptide having the deduced amino acid sequence of FIG. 1 or the polypeptide encoded by the cDNA of the deposited clone. The variant of the polynucleotide may be a naturally occurring allelic variant of the polynucleotide or a non-naturally occurring variant of the polynucleotide.

Thus, the present invention includes polynucleotides encoding the same mature polypeptide as shown in FIG. 1 or the same mature polypeptide encoded by the cDNA of the deposited clone as well as variants of such polynucleotides which variants encode for an fragment, derivative or analog of the polypeptide of FIG. 1 or the polypeptide encoded by the cDNA of the deposited clone. Such nucleotide variants include deletion variants, substitution variants and addition or insertion variants.

As hereinabove indicated, the polynucleotide may have a coding sequence which is a naturally occurring allelic variant of the coding sequence shown in SEQ ID NO:1 or of the coding sequence of the deposited clone. As known in the art, an allelic variant is an alternate form of a polynucleotide sequence which have a substitution, deletion or addition of one or more nucleotides, which does not substantially alter the function of the encoded polypeptide.

The present invention also includes polynucleotides, wherein the coding sequence for the mature polypeptide may be fused in the same reading frame to a polynucleotide which aids in expression and secretion of a polypeptide from a host cell, for example, a leader sequence which functions as a secretory sequence for controlling transport of a

polypeptide from the cell. The polypeptide having a leader sequence is a preprotein and may have the leader sequence cleaved by the host cell to form the mature form of the polypeptide. The polynucleotides may also encode for a proprotein which is the mature protein plus additional 5' amino acid residues. A mature protein having a prosequence is a proprotein and is an inactive form of the protein. Once the prosequence is cleaved an active mature protein remains.

Thus, for example, the polynucleotide of the present invention may encode for a mature protein, or for a protein having a prosequence or for a protein having both a prosequence and presequence (leader sequence).

The polynucleotides of the present invention may also have the coding sequence fused in frame to a marker sequence which allows for purification of the polypeptide of the present invention. The marker sequence may be a hexa-histidine tag supplied by a pQE-9 vector to provide for purification of the mature polypeptide fused to the marker in the case of a bacterial host, or, for example, the marker sequence may be a hemagglutinin (HA) tag when a mammalian host, e.g. COS-7 cells, is used. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein (Wilson, I., et al., Cell, 37:767 (1984)).

The present invention further relates to polynucleotides which hybridize to the hereinabove-described sequences if there is at least 50% and preferably 70% identity between the sequences. The present invention particularly relates to polynucleotides which hybridize under stringent conditions to the hereinabove-described polynucleotides. As herein used, the term "stringent conditions" means hybridization will occur only if there is at least 95% and preferably at least 97% identity between the sequences. The polynucleotides which hybridize to the hereinabove described polynucleotides in a preferred embodiment encode polypeptides which retain substantially the same biological function or activity as the mature polypeptide encoded by the cDNA of SEQ ID NO:2 or the deposited cDNA.

The deposit(s) referred to herein will be maintained under the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the purposes of Patent Procedure. These deposits are provided merely as a convenience and are not an admission that a deposit is required under 35 U.S.C. § 112. The sequence of the polynucleotides contained in the deposited materials, as well as the amino acid sequence of the polypeptides encoded thereby, are incorporated herein by reference and are controlling in the event of any conflict with the description of sequences herein. A license may be required to make, use or sell the deposited materials, and no such license is hereby granted.

The present invention further relates to a VEGF2 polypeptide which has the deduced amino acid sequence of SEQ ID NO:2 or which has the amino acid sequence encoded by the deposited cDNA, as well as fragments, analogs and derivatives of such polypeptide.

The terms "fragment," "derivative" and "analog" when referring to the polypeptide of SEQ ID NO:2 or that encoded by the deposited cDNA, means a polypeptide which retains essentially the same biological function or activity as such polypeptide. Thus, an analog includes a proprotein which can be activated by cleavage of the proprotein portion to produce an active mature polypeptide.

The polypeptide of the present invention may be a recombinant polypeptide, a natural polypeptide or a synthetic polypeptide, preferably a recombinant polypeptide.

The fragment, derivative or analog of the polypeptide of SEQ ID NO:2 or that encoded by the deposited cDNA may

be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or (iv) one in which the additional amino acids are fused to the mature polypeptide, such as a leader or secretory sequence or a sequence which is employed for purification of the mature polypeptide or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

The polypeptides and polynucleotides of the present invention are preferably provided in an isolated form, and preferably are purified to homogeneity.

The term "isolated" means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or DNA or polypeptide, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotide could be part of a vector and/or such polynucleotide or polypeptide could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

The present invention also relates to vectors which include polynucleotides of the present invention, host cells which are genetically engineered with vectors of the invention and the production of polypeptides of the invention by recombinant techniques.

Host cells are genetically engineered (transduced or transformed or transfected) with the vectors of this invention which may be, for example, a cloning vector or an expression vector. The vector may be, for example, in the form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying the VEGF2 genes. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The polynucleotide of the present invention may be employed for producing a polypeptide by recombinant techniques. Thus, for example, the polynucleotide sequence may be included in any one of a variety of expression vehicles, in particular vectors or plasmids for expressing a polypeptide. Such vectors include chromosomal, nonchromosomal and synthetic DNA sequences, e.g., derivatives of SV40; bacterial plasmids; phage DNA; yeast plasmids; vectors derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. However, any other plasmid or vector may be used as long as it is replicable and viable in the host.

As hereinabove described, the appropriate DNA sequence may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease sites by procedures known in the art. Such procedures and others are deemed to be within the scope of those skilled in the art.

The DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s)



(promoter) to direct mRNA synthesis. As representative examples of such promoters, there may be mentioned: LTR or SV40 promoter, the *E. coli* lac or trp, the phage lambda  $P_L$  promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. The expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression.

In addition, the expression vectors preferably contain a gene to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in *E. coli*.

The vector containing the appropriate DNA sequence as herein above described, as well as an appropriate promoter or control sequence, may be employed to transform an appropriate host to permit the host to express the protein. As representative examples of appropriate hosts, there may be mentioned: bacterial cells, such as *E. coli*, *Salmonella typhimurium*, *Streptomyces*; fungal cells, such as yeast; insect cells, such as *Drosophila* and Sf9; animal cells such as CHO, COS or Bowes melanoma; plant cells, etc. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

More particularly, the present invention also includes recombinant constructs comprising one or more of the sequences as broadly described above. The constructs comprise a vector, such as a plasmid or viral vector, into which a sequence of the invention has been inserted, in a forward or reverse orientation. In a preferred aspect of this embodiment, the construct further comprises regulatory sequences, including, for example, a promoter, operably linked to the sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available. The following vectors are provided by way of example. Bacterial: pQE70, pQE-9 (Qiagen), pBS, phagescript, PsiX174, pBluescript SK, pBsKS, pNH8a, pNH16a, pNH18a, pNH46a (Stratagene); pTtrc99A, pKK223-3, pKK233-3, pDR540, PRIT5 (Pharmacia). Eukaryotic: pWLneo, pSV2cat, pOG44, pXT1, pSG (Stratagene) pSVK3, pBPV, PMSG, pSVL (Pharmacia). However, any other plasmid or vector may be used as long as they are replicable and viable in the host.

Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) vectors or other vectors with selectable markers. Two appropriate vectors are pKK223-8 and pCM7. Particular named bacterial promoters include lacI, lacZ, T3, T7, gpt, lambda  $P_R$ , PL and trp. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art.

In a further embodiment, the present invention relates to host cells containing the above-described construct. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation (Davis, L., Dibner, M., Battey, I., Basic Methods in Molecular Biology, 1986)).

The constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence. Alternatively, the polypeptides of the

invention can be synthetically produced by conventional peptide synthesizers.

Mature proteins can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook, et al., Molecular Cloning: A Laboratory Manual, Second Edition, (Cold Spring Harbor, N.Y., 1989), the disclosure of which is hereby incorporated by reference.

Transcription of a DNA encoding the polypeptides of the present invention by higher eukaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Examples include the SV40 enhancer on the late side of the replication origin (bp 100 to 270), a cytomegalovirus early promoter enhancer, a polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, e.g., the ampicillin resistance gene of *E. coli* and *S. cerevisiae* TRP1 gene, and a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK),  $\alpha$ -factor, acid phosphatase, or heat shock proteins, among others. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein into the periplasmic space or extracellular medium. Optionally, the heterologous sequence can encode a fusion protein including an N-terminal identification peptide imparting desired characteristics, e.g., stabilization or simplified purification of expressed recombinant product.

Useful expression vectors for bacterial use are constructed by inserting a structural DNA sequence encoding a desired protein together with suitable translation initiation and termination signals in operable reading phase with a functional promoter. The vector will comprise one or more phenotypic selectable markers and an origin of replication to ensure maintenance of the vector, and to, if desirable, provide amplification within the host. Suitable prokaryotic hosts for transformation include *E. coli*, *Bacillus subtilis*, *Salmonella typhimurium* and various species within the genera *Pseudomonas*, *Streptomyces*, and *Staphylococcus*, although others may also be employed as a matter of choice.

As a representative but nonlimiting example, useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and GEM1 (Promega Biotec, Madison, Wis., USA). These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed.

Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter is derepressed by appropriate means (e.g., temperature shift or chemical induction) and cells are cultured for an additional period.

Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification.

Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents.

Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described by Gluzman, *Cell*, 23:175 (1981), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA sequences derived from the SV40 viral genome, for example, SV40 origin, early promoter, enhancer, splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements.

VEGF2 is recovered and purified from recombinant cell cultures by methods used heretofore, including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxyapatite chromatography and lectin chromatography. It is preferred to have low concentrations (approximately 0.1–5 mM) of calcium ion present during purification (Price, et al., *J. Biol. Chem.*, 244:917 (1969)). Protein refolding steps can be used, as necessary, in completing configuration of the mature protein. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps.

The polypeptides of the present invention may be a naturally purified product, or a product of chemical synthetic procedures, or produced by recombinant techniques from a prokaryotic or eukaryotic host (for example, by bacterial, yeast, higher plant, insect and mammalian cells in culture). Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated with mammalian or other eukaryotic carbohydrates or may be non-glycosylated.

VEGF2 is useful as a wound healing agent, particularly where it is necessary to re-vascularize damaged tissues, or where new capillary angiogenesis is important. Therefore, it may be used for treatment of full-thickness wounds such as dermal ulcers, including pressure sores, venous ulcers, and diabetic ulcers. In addition, it can be used in the treatment of full-thickness burns and injuries where angiogenesis is desired to prepare the burn or injured sites for a skin graft and flap. In this case, it should be applied directly at the sites. Similar, VEGF2 can be used in plastic surgery when reconstruction is required following a burn, other trauma, or even for cosmetic purposes.

VEGF2 may also be used to induce the growth of damaged bone, periodontium or ligament tissue. It may be used in periodontal disease where VEGF2 is applied in a methylcellulose gel to the roots of the diseased teeth, the treatment could lead to the formation of new bone and cementum with collagen fiber ingrowths. It can be used for regenerating supporting tissues of teeth, including alveolar bone, cementum and periodontal ligament, that have been damaged by disease and trauma.

Since angiogenesis is important in keeping wounds clean and non-infected, VEGF2 may be used in association with

surgery and following the repair of cuts. It should be particularly useful in the treatment of abdominal wounds where there is a high risk of infection.

VEGF2 can be used for the promotion of endothelialization in vascular graft surgery. In the case of vascular grafts using either transplanted or synthetic material, VEGF2 can be applied to the surface of the graft or at the junction to promote the growth of the vascular endothelial cells. One derivation of this is that VEGF2 can be used to repair the damage of myocardial infarction and other occasions where coronary bypass surgery is needed by stimulating the growth of the transplanted tissue. Related to this is the use of VEGF2 to repair the cardiac vascular system after ischemia.

The identification of VEGF2 can be used for the generation of certain inhibitors of vascular endothelial growth factor. Since angiogenesis and neovascularization are essential steps in solid tumor growth, inhibition of angiogenic activity of the vascular endothelial growth factor is very useful to prevent the further growth, retard, or even regress solid tumors. Although the level of expression of VEGF2 is extremely low in normal tissues including breast, it can be found expressed at moderate levels in at least two breast tumor cell lines that are derived from malignant tumors. It is, therefore, possible that VEGF2 is involved in tumor angiogenesis and growth.

VEGF2 can be used for in vitro culturing of vascular endothelial cells, where it can be added to the conditional medium to a concentration from 10 pg/ml to 10 ng/ml.

The polypeptide of the present invention may also be employed in accordance with the present invention by expression of such polypeptide in vivo, which is often referred to as "gene therapy."

Thus, for example, cells such as bone marrow cells may be engineered with a polynucleotide (DNA or RNA) encoding for the polypeptide ex vivo, the engineered cells are then provided to a patient to be treated with the polypeptide. Such methods are well-known in the art. For example, cells may be engineered by procedures known in the art by use of a retroviral particle containing RNA encoding for the polypeptide of the present invention.

Similarly, cells may be engineered in vivo for expression of the polypeptide in vivo, for example, by procedures known in the art. As known in the art, a producer cell for producing a retroviral particle containing RNA encoding the polypeptide of the present invention may be administered to a patient for engineering cells in vivo and expression of the polypeptide in vivo. These and other methods for administering a polypeptide of the present invention by such methods should be apparent to those skilled in the art from the teachings of the present invention. For example, the expression vehicle for engineering cells may be other than a retroviral particle, for example, an adenovirus, which may be used to engineering cells in vivo after combination with a suitable delivery vehicle.

The polypeptide of the present invention may be employed in combination with a suitable pharmaceutical carrier. Such compositions comprise a therapeutically effective amount of the protein, and a pharmaceutically acceptable carrier or excipient. Such a carrier includes but is not limited to saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The formulation should suit the mode of administration.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Associated with such container(s) can be a notice

in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. In addition, the polypeptide of the present invention may be employed on conjunction with other therapeutic compounds.

The pharmaceutical compositions may be administered in a convenient manner, such as the oral, and intravenous routes, and is preferably administered topically. The amounts and dosage regimens of VEGF2 administered to a subject will depend on a number of factors, such as the mode of administration, the nature of the condition being treated, the body weight of the subject being treated and the judgment of the prescribing physician. Generally speaking, it is given, for example, in therapeutically effective doses of at least about 10  $\mu\text{g/kg}$  body weight and, in most cases, it would be administered in an amount not in excess of about 8  $\text{mg/kg}$  body weight per day and preferably the dosage is from about 10  $\mu\text{g/kg}$  body weight to about 1  $\text{mg/kg}$  body weight daily, taking into the account the routes of administration, symptoms, etc.

The sequences of the present invention are also valuable for chromosome identification. The sequence is specifically targeted to and can hybridize with a particular location on an individual human chromosome. Moreover, there is a current need for identifying particular sites on the chromosome. Few chromosome marking reagents based on actual sequence data (repeat polymorphism's) are presently available for marking chromosomal location. The mapping of DNAs to chromosomes according to the present invention is an important first step in correlating those sequences with genes associated with disease.

Briefly, sequences can be mapped to chromosomes by preparing PCR primers (preferably 15–25 bp) from the cDNA. Computer analysis of the cDNA is used to rapidly select primers that do not span more than one exon in the genomic DNA, thus complicating the amplification process. These primers are then used for PCR screening of somatic cell hybrids containing individual human chromosomes. Only those hybrids containing the human gene corresponding to the primer will yield an amplified fragment.

PCR mapping of somatic cell hybrids is a rapid procedure for assigning a particular DNA to a particular chromosome. Using the present invention with the same oligonucleotide primers, sublocalization can be achieved with panels of fragments from specific chromosomes or pools of large genomic clones in an analogous manner. Other mapping strategies that can similarly be used to map to its chromosome include in situ hybridization, prescreening with labeled flow-sorted chromosomes and preselection by hybridization to construct chromosome specific-cDNA libraries.

Fluorescence in situ hybridization (FISH) of a cDNA clone to a metaphase chromosomal spread can be used to provide a precise chromosomal location in one step. This technique can be used with cDNA as short as 500 or 600 bases; however, clones larger than 2,000 bp have a higher likelihood of binding to a unique chromosomal location with sufficient signal intensity for simple detection. FISH requires use of the clone from which the EST was derived, and the longer the better. For example, 2,000 bp is good, 4,000 is better, and more than 4,000 is probably not necessary to get good results a reasonable percentage of the time. For a review of this technique, see Verma et al., *Human Chromosomes: a Manual of Basic Techniques*. Pergamon Press, New York (1988).

Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. (Such data are found, for example, in V. McKusick, Mendelian Inheritance in Man (available on line through Johns Hopkins University Welch Medical Library). The relationship between genes and diseases that have been mapped to the same chromosomal region are then identified through linkage analysis (coinheritance of physically adjacent genes).

Next, it is necessary to determine the differences in the cDNA or genomic sequence between affected and unaffected individuals. If a mutation is observed in some or all of the affected individuals but not in any normal individuals, then the mutation is likely to be the causative agent of the disease.

With current resolution of physical mapping and genetic mapping techniques, a cDNA precisely localized to a chromosomal region associated with the disease could be one of between 50 and 500 potential causative genes. (This assumes 1 megabase mapping resolution and one gene per 20 kb).

Comparison of affected and unaffected individuals generally involves first looking for structural alterations in the chromosomes, such as deletions or translocations that are visible from chromosome spreads or detectable using PCR based on that cDNA sequence. Ultimately, complete sequencing of genes from several individuals is required to confirm the presence of a mutation and to distinguish mutations from polymorphisms.

The present invention is further directed to inhibiting VEGF2 in vivo by the use of antisense technology. Antisense technology can be used to control gene expression through triple-helix formation or antisense DNA or RNA, both of which methods are based on binding of a polynucleotide to DNA or RNA. For example, the 5' coding portion of the mature polynucleotide sequence, which encodes for the polypeptide of the present invention, is used to design an antisense RNA oligonucleotide of from 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription (triple helix—see Lee et al, *Nucl. Acids Res.*, 6:3073 (1979); Cooney et al, *Science*, 241:456 (1988); and Dervan et al, *Science*, 251: 1360 (1991), thereby preventing transcription and the production of VEGF2. The antisense RNA oligonucleotide hybridizes to the mRNA in vivo and blocks translation of an mRNA molecule into the VEGF2 (antisense—Okano, *J. Neurochem.*, 56:560 (1991); *Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression*, CRC Press, Boca Raton, Fla. (1988)).

Alternatively, the oligonucleotides described above can be delivered to cells by procedures in the art such that the anti-sense RNA or DNA may be expressed in vivo to inhibit production of VEGF2 in the manner described above.

Antisense constructs to VEGF2, therefore, may inhibit the angiogenic activity of the VEGF2 and prevent the further growth or even regress solid tumors, since angiogenesis and neovascularization are essential steps in solid tumor growth. These antisense constructs may also be used to treat rheumatoid arthritis, psoriasis and diabetic retinopathy which are all characterized by abnormal angiogenesis.

The polypeptides, their fragments or other derivatives, or analogs thereof, or cells expressing them can be used as an immunogen to produce antibodies thereto. These antibodies can be, for example, polyclonal or monoclonal antibodies. The present invention also includes chimeric, single chain, and humanized antibodies, as well as Fab fragments, or the product of an Fab expression library. Various procedures

known in the art may be used for the production of such antibodies and fragments.

Antibodies generated against the polypeptide corresponding to a sequence of the present invention can be obtained by direct injection of the polypeptide into an animal or by administering the polypeptide to an animal, preferably a nonhuman. The antibody so obtained will then bind the polypeptide itself. In this manner, even a sequence encoding only a fragment of the polypeptide can be used to generate antibodies binding the whole native polypeptide. Such antibodies can then be used to isolate the polypeptide from tissue expressing that polypeptide.

For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique (Kohler and Milstein, 1975, *Nature*, 256:495-497), the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, *Immunology Today* 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Colé, et al., 1985, in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96).

Techniques described for the production of single chain antibodies (U.S. Pat. No. 4,946,778) can be adapted to produce single chain antibodies to immunogenic polypeptide products of this invention.

Neutralization antibodies can be identified and applied to mask the vascular endothelial growth factor, and that has been shown in mice model systems against VEGF. VEGF2 can also be inactivated by certain dominant negative mutants within the gene itself. It is known that both PDGF $\alpha$  and  $\beta$  form either heterodimers or homodimers, and VEGF forms homodimers. Similar interaction between VEGF2 could be expected. These antibodies therefore may be used to block the angiogenic activity of VEGF2 and retard the growth of solid tumors. These antibodies may also be used to treat inflammation caused by the increased vascular permeability which results from the presence of VEGF2.

These antibodies may further be used in an immunoassay to detect the presence of tumors in certain individuals. Enzyme immunoassay can be performed from the blood sample of an individual. Elevated levels of VEGF2 can be considered diagnostic of cancer.

The present invention is also directed to antagonist/inhibitors of the polypeptides of the present invention. The antagonist/inhibitors are those which inhibit or eliminate the function of the polypeptide.

Thus, for example, antagonists bind to a polypeptide of the present invention and inhibit or eliminate its function. The antagonist, for example, could be an antibody against the polypeptide which binds to the polypeptide or, in some cases, an oligonucleotide. An example of an inhibitor is a small molecule which binds to and occupies the catalytic site of the polypeptide thereby making the catalytic site inaccessible to substrate such that normal biological activity is prevented. Examples of small molecules include but are not limited to small peptides or peptide-like molecules.

Truncated versions of VEGF2 can also be produced that are capable of interacting with wild type VEGF2 form dimers that fail activate endothelial cell growth, therefore inactivated the endogenous VEGF2. Or, mutant forms of VEGF2 form dimers themselves and occupies the ligand binding domain of the proper tyrosine Kinase receptors on the target cell surface, but fail to activate the cell growth.

Alternatively, antagonists to the polypeptides of the present invention may be employed which bind to the

receptors to which a polypeptide of the present invention normally binds. The antagonists may be closely related proteins such that they recognize and bind to the receptor sites of the natural protein, however, they are inactive forms of the natural protein and thereby prevent the action of VEGF2 since receptor sites are occupied. In these ways, the action of the VEGF2 is prevented and the antagonist/inhibitors may be used therapeutically as an anti-tumor drug by occupying the receptor sites of tumors which are recognized by VEGF2 or by inactivating VEGF2 itself. The antagonist/inhibitors may also be used to prevent inflammation due to the increased vascular permeability action of VEGF2. The antagonist/inhibitors may also be used to treat solid tumor growth, diabetic retinopathy, psoriasis and rheumatoid arthritis.

The antagonist/inhibitors may be employed in a composition with a pharmaceutically acceptable carrier, e.g., as hereinabove described.

The present invention will be further described with reference to the following examples; however, it is to be understood that the present invention is not limited to such examples. All parts or amounts, unless otherwise specified, are by weight.

In order to facilitate understanding of the following examples, certain frequently occurring methods and/or terms will be described.

"Plasmids" are designated by a lower case p preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are either commercially available, publicly available on an unrestricted basis, or can be constructed from available plasmids in accord with published procedures. In addition, equivalent plasmids to those described are known in the art and will be apparent to the ordinarily skilled artisan.

"Digestion" of DNA refers to catalytic cleavage of the DNA with a restriction enzyme that acts only at certain sequences in the DNA. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors and other requirements were used as would be known to the ordinarily skilled artisan. For analytical purposes, typically 1  $\mu$ g of plasmid or DNA fragment is used with about 2 units of enzyme in about 20  $\mu$ l of buffer solution. For the purpose of isolating DNA fragments for plasmid construction, typically 5 to 50  $\mu$ g of DNA are digested with 20 to 250 units of enzyme in a larger volume. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation times of about 1 hour at 37° C. are ordinarily used, but may vary in accordance with the supplier's instructions. After digestion the reaction is electrophoresed directly on a poly-acrylamide gel to isolate the desired fragment.

Size separation of the cleaved fragments is performed using 8 percent polyacrylamide gel described by Goeddel, D. et al., *Nucleic Acids Res.*, 8:4057 (1980).

"Oligonucleotides" refers to either a single stranded polydeoxynucleotide or two complementary polydeoxynucleotide strands which may be chemically synthesized. Such synthetic oligonucleotides have no 5' phosphate and thus will not ligate to another oligonucleotide without adding a phosphate with an ATP in the presence of a kinase. A synthetic oligonucleotide will ligate to a fragment that has not been dephosphorylated.

"Ligation" refers to the process of forming phosphodiester bonds between two double stranded nucleic acid fragments (Maniatis, T., et al., *Id.*, p. 146). Unless otherwise provided, ligation may be accomplished using known buff-

ers and conditions with 10 units of T4 DNA ligase ("ligase") per 0.5 µg of approximately equimolar amounts of the DNA fragments to be ligated.

Unless otherwise stated, transformation was performed as described by the method of Graham, F. and Van der Eb, A., *Virology*, 52:456-457 (1973).

#### EXAMPLE 1

Expression, pattern of VEGF2 in human tissues and breast cancer cell lines

Northern blot analysis was carried out to examine the levels of expression of VEGF2 in human tissues and breast cancer cell lines in human tissues. Total cellular RNA samples were isolated with RNazol™ B system (Biotecx Laboratories, Inc.). About 10 µg of total RNA isolated from each breast tissue and cell line specified was separated on 1% agarose gel and blotted onto a nylon filter, (Molecular Cloning, Sambrook Fritsch, and Maniatis, Cold Spring Harbor Press, 1989). The labeling reaction was done according to the Stratagene Prime-It kit with 50 ng DNA fragment. The labeled DNA was purified with a Select-G-50 column from 5' Prime-3 Prime, Inc. The filter was then hybridized with radioactive labeled full length VEGF2 gene at 1,000,000 cpm/ml in 0.5 M NaPO<sub>4</sub> and 7% SDS overnight at 65° C. After wash twice at room temperature and twice at 60° C. with 0.5 X SSC, 0.1% SDS, the filters were then exposed at -70° C. overnight with intensifying screen. A message of 1.6 Kb was observed in 2 breast cancer cell lines. Lane #4 represents a very tumorigenic cell line that is estrogen independent for growth. See FIG. 4. Also, 10 µg of total RNA from 10 human adult tissues were separated on an agarose gel and blotted onto a nylon filter. The filter was then hybridized with radioactively labeled VEGF2 probe in 7% SDS, 0.5 M NaPO<sub>4</sub>, pH 7.2; 1% BSA overnight at 65° C. Following was in 0.2 X SSC at 65° C., the filter was exposed to film for 24 days at -70° C. with intensifying screen. See FIG. 5.

#### EXAMPLE 2

Expression of VEGF2 by in vitro transcript I on and translation

The VEGF2 cDNA was transcribed and translated in vitro to determine the size of the translatable polypeptide encoded by the full length and partial VEGF2 cDNA. The full length and partial cDNA inserts of VEGF2 in the pBluescript SK vector were amplified by PCR with three pairs of primers, 1) M13-reverse and forward primers; 2) M13-reverse primer and VEGF primer F4; 3) M13-reverse primer and VEGF primer F5. The sequence of these primers are as follows.

M13-2 reverse primer:

5'-ATGCTTCCGGCTCGTATG-3' (SEQ ID NO:4)

This sequence is located upstream of the 5' end of the VEGF2 cDNA insert in the pBluescript vector and is in an anti-sense orientation as the cDNA. A T3 promoter sequence is located between this primer and the VEGF2 cDNA.

M13-2 forward primer:

5'-GGGTTTTCCAGTCACGAC-3' (SEQ ID NO:5)

This sequence is located downstream of the 3' end of the VEGF2 cDNA insert in the pBluescript vector and is in an anti-sense orientation as the cDNA insert.

VEGF primer F4:

5'-CCACATGGTTCAGGAAAGACA-3' (SEQ ID NO:6)

This sequence is located within the VEGF2 cDNA in an anti-sense orientation from bp 1259-1239, which is about 169 bp away from the 3' end of the stop codon and about 266 bp before the last nucleotide of the cDNA.

PCR reaction with all three pairs of primers produce amplified products with T3 promoter sequence in front of the cDNA insert. The first and third pairs of primers produce PCR products that encode the full polypeptide of VEGF2. The second pair of primers produce PCR product that misses 36 amino acids coding sequence at the C-terminus of the VEGF2 polypeptide.

Approximately 0.5 µg of PCR product from first pair of primers, 1 µg from second pair of primers, 1 µg from third pair of primers were used for in vitro transcription/translation. The in vitro transcription/translation reaction was performed in a 25 µl of volume, using the T<sub>N</sub>T™ Coupled Reticulocyte Lysate Systems (promega, CAT# L4950). Specifically, the reaction contains 12.5 µl of TNT rabbit reticulocyte lysate 2 µl of TNT reaction buffer, 1 µl of T3 polymerase, 1 µl of 1 mM amino acid mixture (minus methionine), 4 µl of <sup>35</sup>S-methionine (>1000 Ci/mmol, 10 mCi/ml), 1 µl of 40 U/µl; RNasin ribonuclease inhibitor, 0.5 or 1 µg of PCR products. Nuclease-free H<sub>2</sub>O were added to bring the me to 25 µl. The reaction was incubated at 30° C. for 2 hours. Five microliters of the reaction product was analyzed on a 4-20% gradient SDS-PAGE gel. After fixing in 25% isopropanol and 10% acetic acid, the gel was dried and exposed to an X-ray film overnight at 70° C.

As shown in FIG. 6, PCR products containing the full length VEGF2 cDNA and the cDNA missing 266 bp in the 3' untranslated region (3'-UTR) produced the same length of translated products, whose molecular weights are estimated to be 38-40 dk (lanes 1 & 3). The cDNA missing all the 3'UTR and missing sequence encoding the C-terminal 36 amino acids was translated into a polypeptide with an estimated molecular weight of 36-38 kd (lane 2).

#### SEQUENCE LISTING

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 -10 -5 -1 1 5  
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 Ala Thr Asn Thr Phe Phe Lys Pro Pro Cys Val Ser Val Tyr Arg Cys  
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 Ser Tyr Leu Ser Lys Thr Leu Phe Glu Ile Thr Val Pro Leu Ser Gln  
 90 95 100  
 ggc ccc aaa cca gta aca atc agt ttt gcc aat cac act tcc tgc cga 493  
 Gly Pro Lys Pro Val Thr Ile Ser Phe Ala Asn His Thr Ser Cys Arg  
 105 110 115  
 tgc atg tct aaa ctg gat gtt tac aga caa gtt cat tcc att att aga 541  
 Cys Met Ser Lys Leu Asp Val Tyr Arg Gln Val His Ser Ile Ile Arg  
 120 125 130  
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 His Lys Glu Leu Asp Arg Asn Ser Cys Gln Cys Val Cys Lys Asn Lys  
 215 220 225  
 ctc ttc ccc agc caa tgt ggg gcc aac cga gaa ttt gat gaa aac aca 877  
 Leu Phe Pro Ser Gln Cys Gly Ala Asn Arg Glu Phe Asp Glu Asn Thr  
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 10 15 20

Ile Leu Lys Ser Ile Asp Asn Glu Trp Arg Lys Thr Gln Cys Met Pro  
 25 30 35 40

Arg Glu Val Cys Ile Asp Val Gly Lys Glu Phe Gly Val Ala Thr Asn  
 45 50 55

Thr Phe Phe Lys Pro Pro Cys Val Ser Val Tyr Arg Cys Gly Gly Cys  
 60 65 70

Cys Asn Ser Glu Gly Leu Gln Cys Met Asn Thr Ser Thr Ser Tyr Leu  
 75 80 85

Ser Lys Thr Leu Phe Glu Ile Thr Val Pro Leu Ser Gln Gly Pro Lys  
 90 95 100

Pro Val Thr Ile Ser Phe Ala Asn His Thr Ser Cys Arg Cys Met Ser  
 105 110 115 120

Lys Leu Asp Val Tyr Arg Gln Val His Ser Ile Ile Arg Arg Ser Leu  
 125 130 135

Pro Ala Thr Leu Pro Gln Cys Gln Ala Ala Asn Lys Thr Cys Pro Thr  
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Asn Tyr Met Trp Asn Asn His Ile Cys Arg Cys Leu Ala Gln Glu Asp  
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Phe Met Phe Ser Ser Asp Ala Gly Asp Asp Ser Thr Asp Gly Phe His  
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Asp Ile Cys Gly Pro Asn Lys Glu Leu Asp Glu Glu Thr Cys Gln Cys  
 185 190 195 200

Val Cys Arg Ala Gly Leu Arg Pro Ala Ser Cys Gly Pro His Lys Glu  
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Ser Gln Cys Gly Ala Asn Arg Glu Phe Asp Glu Asn Thr Cys Gln Cys  
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Val Cys Lys Arg Thr Cys Pro Arg Asn Gln Pro Leu Asn Pro Gly Lys  
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Cys Ala Cys Glu Cys Thr Glu Ser Pro Gln Lys Cys Leu Leu Lys Gly  
265 270 275 280

Lys Lys Phe His His Gln Thr Cys Ser Cys Tyr Arg Arg Pro Cys Thr  
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 20 25 30  
 Leu Ala Arg Ser Gln Ile His Ser Ile Arg Asp Leu Gln Arg Leu Leu  
 35 40 45  
 Glu Ile Asp Ser Val Gly Ser Glu Asp Ser Leu Asp Thr Ser Leu Arg  
 50 55 60  
 Ala His Gly Val His Ala Thr Lys His Val Pro Glu Lys Arg Pro Leu  
 65 70 75 80  
 Pro Ile Arg Arg Lys Arg Ser Ile Glu Glu Ala Val Pro Ala Val Cys  
 85 90 95  
 Lys Thr Arg Thr Val Ile Tyr Glu Ile Pro Arg Ser Gln Val Asp Pro  
 100 105 110  
 Thr Ser Ala Asn Phe Leu Ile Trp Pro Pro Cys Val Glu Val Lys Arg  
 115 120 125  
 Cys Thr Gly Cys Cys Asn Thr Ser Ser Val Lys Cys Gln Pro Ser Arg  
 130 135 140  
 Val His His Arg Ser Val Lys Val Ala Lys Val Glu Tyr Val Arg Lys  
 145 150 155 160  
 Lys Pro Lys Leu Lys Glu Val Gln Val Arg Leu Glu Glu His Leu Glu  
 165 170 175  
 Cys Ala Cys Ala Thr Thr Ser Leu Asn Pro Asp Tyr Arg Glu Glu Asp  
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&lt;211&gt; LENGTH: 241

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Homo sapiens

&lt;400&gt; SEQUENCE: 8

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 1 5 10 15  
 Leu Val Ser Ala Glu Gly Asp Pro Ile Pro Glu Glu Leu Tyr Glu Met  
 20 25 30  
 Leu Ser Asp His Ser Ile Arg Ser Phe Asp Asp Leu Gln Arg Leu Leu  
 35 40 45  
 His Gly Asp Pro Gly Glu Glu Asp Gly Ala Glu Leu Asp Leu Asn Met  
 50 55 60  
 Thr Arg Ser His Ser Gly Gly Glu Leu Glu Ser Leu Ala Arg Gly Arg  
 65 70 75 80  
 Arg Ser Leu Gly Ser Leu Thr Ile Ala Glu Pro Ala Met Ile Ala Glu  
 85 90 95  
 Cys Lys Thr Arg Thr Glu Val Phe Glu Ile Ser Arg Arg Leu Ile Asp  
 100 105 110  
 Arg Thr Asn Ala Asn Phe Leu Val Trp Pro Pro Cys Val Glu Val Gln  
 115 120 125  
 Arg Cys Ser Gly Cys Cys Asn Asn Arg Asn Val Gln Cys Arg Pro Thr  
 130 135 140  
 Gln Val Gln Leu Arg Pro Val Gln Val Arg Lys Ile Glu Ile Val Arg  
 145 150 155 160

-continued

Lys Lys Pro Ile Phe Lys Lys Ala Thr Val Thr Leu Glu Asp His Leu  
165 170 175  
Ala Cys Lys Cys Glu Thr Val Ala Ala Ala Arg Pro Val Thr Arg Ser  
180 185 190  
Pro Gly Gly Ser Gln Glu Gln Arg Ala Lys Thr Pro Gln Thr Arg Val  
195 200 205  
Thr Ile Arg Thr Val Arg Val Arg Arg Pro Pro Lys Gly Lys His Arg  
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20 25 30  
Gly Gly Gln Asn His His Glu Val Val Lys Phe Met Asp Val Tyr Gln  
35 40 45  
Arg Ser Tyr Cys His Pro Ile Glu Thr Leu Val Asp Ile Phe Gln Glu  
50 55 60  
Tyr Pro Asp Glu Ile Glu Tyr Ile Phe Lys Pro Ser Cys Val Pro Leu  
65 70 75 80  
Met Arg Cys Gly Gly Cys Cys Asn Asp Glu Gly Leu Glu Cys Val Pro  
85 90 95  
Thr Glu Glu Ser Asn Ile Thr Met Gln Ile Met Arg Ile Lys Pro His  
100 105 110  
Gln Gly Gln His Ile Gly Glu Met Ser Phe Leu Gln His Asn Lys Cys  
115 120 125  
Glu Cys Arg Pro Lys Lys Asp Arg Ala Arg Gln Glu Lys Lys Ser Val  
130 135 140  
Arg Gly Lys Gly Lys Gly Gln Lys Arg Lys Arg Lys Lys Ser Arg Tyr  
145 150 155 160  
Lys Ser Trp Ser Val Tyr Val Gly Ala Arg Cys Cys Leu Met Pro Trp  
165 170 175  
Ser Leu Pro Gly Pro His Pro Cys Gly Pro Cys Ser Glu Arg Arg Lys  
180 185 190  
His Leu Phe Val Gln Asp Pro Gln Thr Cys Lys Cys Ser Cys Lys Asn  
195 200 205  
Thr Asp Ser Arg Cys Lys Ala Arg Gln Leu Glu Lys Asn Glu Arg Thr  
210 215 220  
Cys Arg Cys Asp Lys Pro Arg Arg  
225 230

What is claimed is:

1. An isolated polynucleotide encoding a mature portion of a protein consisting of SEQ ID NO:2.
2. The isolated polynucleotide of claim 1 encoding a proprotein portion of a protein consisting of SEQ ID NO:2.
3. The polynucleotide of claim 2 fused to a polynucleotide which encodes a heterologous polypeptide.

4. A recombinant vector comprising the polynucleotide of claim 2.
5. A recombinant vector comprising the polynucleotide of claim 2 operatively associated with a regulatory sequence that controls gene expression.
6. A genetically engineered host cell comprising the polynucleotide of claim 2.

7. A genetically engineered host cell comprising the polynucleotide of claim 2 operatively associated with a heterologous regulatory sequence that controls gene expression.

8. A method for producing a VEGF-2 polypeptide, comprising:

(a) culturing the genetically engineered host cell of claim 7 under conditions suitable to produce the polypeptide; and

(b) recovering the polypeptide from the cell culture.

9. The polynucleotide of claim 2 fused to a heterologous polynucleotide.

10. The polynucleotide of claim 1 fused to a polynucleotide which encodes a heterologous polypeptide.

11. A recombinant vector comprising the polynucleotide of claim 1.

12. A recombinant vector comprising the polynucleotide of claim 1 operatively associated with a regulatory sequence that controls gene expression.

13. A genetically engineered host cell comprising the polynucleotide of claim 1.

14. A genetically engineered host cell comprising the polynucleotide of claim 1 operatively associated with a heterologous regulatory sequence that controls gene expression.

15. A method for producing a VEGF-2 polypeptide, comprising:

(a) culturing the genetically engineered host cell of claim 14 under conditions suitable to produce the polypeptide; and

(b) recovering the polypeptide from the cell culture.

16. The polynucleotide of claim 1 fused to a heterologous polynucleotide.

17. An isolated polynucleotide which encodes a polypeptide comprising amino acids 61-74 of SEQ ID NO:2.

18. The isolated polynucleotide of claim 17, wherein the polypeptide comprises amino acids 1-326 of SEQ ID NO:2.

19. The polynucleotide of claim 18 fused to a polynucleotide which encodes a heterologous polypeptide.

20. A recombinant vector comprising the polynucleotide of claim 18.

21. A recombinant vector comprising the polynucleotide of claim 18 operatively associated with a regulatory sequence that controls gene expression.

22. A genetically engineered host cell comprising the polynucleotide of claim 18.

23. A genetically engineered host cell comprising the polynucleotide of claim 18 operatively associated with a heterologous regulatory sequence that controls gene expression.

24. A method for producing a VEGF-2 polypeptide, comprising:

(a) culturing the genetically engineered host cell of claim 23 under conditions suitable to produce the polypeptide; and

(b) recovering the polypeptide from the cell culture.

25. The polynucleotide of claim 18 fused to a heterologous polynucleotide.

26. The isolated polynucleotide of claim 17, wherein the polypeptide comprises amino acids 24-326 of SEQ ID NO:2.

27. The polynucleotide of claim 26 fused to a polynucleotide which encodes a heterologous polypeptide.

28. A recombinant vector comprising the polynucleotide of claim 26.

29. A recombinant vector comprising the polynucleotide of claim 26 operatively associated with a regulatory sequence that controls gene expression.

30. A genetically engineered host cell comprising the polynucleotide of claim 26.

31. A genetically engineered host cell comprising the polynucleotide of claim 26 operatively associated with a heterologous regulatory sequence that controls gene expression.

32. A method for producing a VEGF-2 polypeptide, comprising:

(a) culturing the genetically engineered host cell of claim 31 under conditions suitable to produce the polypeptide; and

(b) recovering the polypeptide from the cell culture.

33. The polynucleotide of claim 26 fused to a heterologous polynucleotide.

34. The polynucleotide of claim 17 fused to a polynucleotide which encodes a heterologous polypeptide.

35. A recombinant vector comprising the polynucleotide of claim 17.

36. A recombinant vector comprising the polynucleotide of claim 17 operatively associated with a regulatory sequence that controls gene expression.

37. A genetically engineered host cell comprising the polynucleotide of claim 17.

38. A genetically engineered host cell comprising the polynucleotide of claim 17 operatively associated with a heterologous regulatory sequence that controls gene expression.

39. A method for producing a VEGF-2 polypeptide, comprising:

(a) culturing the genetically engineered host cell of claim 38 under conditions suitable to produce the polypeptide; and

(b) recovering the polypeptide from the cell culture.

40. The polynucleotide of claim 17 fused to a heterologous polynucleotide.

41. An isolated polynucleotide which hybridizes to a polynucleotide consisting of a nucleotide sequence of SEQ ID NO: 1 or the complement of SEQ ID NO: 1 under the following conditions: hybridization in 0.5M NaPO<sub>4</sub>, 7% sodium dodecyl sulfate (SDS) at 65° C. and washing with 0.5×SSC, 0.1% SDS at 60° C.

42. The isolated polynucleotide of claim 41, wherein the polynucleotide is DNA.

43. The isolated polynucleotide of claim 41, wherein the polynucleotide is RNA.

44. The polynucleotide of claim 41 fused to a polynucleotide which encodes a heterologous polypeptide.

45. A recombinant vector comprising the polynucleotide of claim 41.

46. A recombinant vector comprising the polynucleotide of claim 41 operatively associated with a regulatory sequence that controls gene expression.

47. A genetically engineered host cell comprising the polynucleotide of claim 41.

48. A genetically engineered host cell comprising the polynucleotide of claim 41 operatively associated with a heterologous regulatory sequence that controls gene expression.

49. The polynucleotide of claim 41 fused to a heterologous polynucleotide.

50. An isolated polynucleotide which hybridizes to the cDNA contained in ATCC Deposit No. 75968 under the following conditions: hybridization in 0.5M NaPO<sub>4</sub>, 7% sodium dodecyl sulfate (SDS) at 65° C. and washing with 0.5×SSC, 0.1% SDS at 60° C.

51. The isolated polynucleotide of claim 50, wherein the polynucleotide is DNA.

52. The isolated polynucleotide of claim 50, wherein the polynucleotide is RNA.

53. The polynucleotide of claim 50 fused to a polynucleotide which encodes a heterologous polypeptide.

54. A recombinant vector comprising the polynucleotide of claim 50.

55. A recombinant vector comprising the polynucleotide of claim 50 operatively associated with a regulatory sequence that controls gene expression.

56. A genetically engineered host cell comprising the polynucleotide of claim 50.

57. A genetically engineered host cell comprising the polynucleotide of claim 50 operatively associated with a heterologous regulatory sequence that controls gene expression.

58. A method for producing a VEGF-2 polypeptide, comprising:

(a) culturing the genetically engineered host cell of claim 57 under conditions suitable to produce the polypeptide; and

(b) recovering the polypeptide from the cell culture.

59. The polynucleotide of claim 50 fused to a heterologous polynucleotide.

60. A method for producing a VEGF-2 polypeptide, comprising:

(a) culturing a genetically engineered host cell comprising an isolated mammalian RNA or cDNA which hybridizes to a polynucleotide consisting of a nucleic sequence or the complement of SEQ ID NO:1 under the following conditions; hybridization in 0.5M NaPO<sub>4</sub> 7% sodium dodecyl sulfate (SDS) at 65° C. and washing with 0.5xSSC 0.1% SDS at 60° C. under conditions suitable to produce the polypeptide; and

(b) recovering the polypeptide from the cell culture.

61. An isolated polynucleotide comprising a polynucleotide that encodes a polypeptide fragment of SEQ ID NO:2, wherein said polypeptide fragment has angiogenic activity.

62. The polynucleotide of claim 61 fused to a heterologous polynucleotide.

63. The polynucleotide of claim 61 fused to a polynucleotide which encodes a heterologous polypeptide.

64. A recombinant vector comprising the polynucleotide of claim 61.

65. A recombinant vector comprising the polynucleotide of claim 61 operatively associated with a regulatory sequence that controls gene expression.

66. A genetically engineered host cell comprising the polynucleotide of claim 61.

67. A genetically engineered host cell comprising the polynucleotide of claim 61 operatively associated with a heterologous regulatory sequence that controls gene expression.

68. A method for producing a VEGF-2 polypeptide, comprising:

(a) culturing the genetically engineered host cell of claim 67 under conditions suitable to produce the polypeptide; and

(b) recovering the polypeptide from the cell culture.

69. An isolated polynucleotide comprising a polynucleotide that encodes a polypeptide fragment encoded by the cDNA contained in ATCC Deposit No. 75968, wherein said fragment has angiogenic activity.

70. The polynucleotide of claim 69 fused to a heterologous polynucleotide.

71. The polynucleotide of claim 69 fused to a polynucleotide which encodes a heterologous polypeptide.

72. A recombinant vector comprising the polynucleotide of claim 69.

73. A recombinant vector comprising the polynucleotide of claim 69 operatively associated with a regulatory sequence that controls gene expression.

74. A genetically engineered host cell comprising the polynucleotide of claim 69.

75. A genetically engineered host cell comprising the polynucleotide of claim 69 operatively associated with a heterologous regulatory sequence that controls gene expression.

76. A method for producing a VEGF-2 polypeptide, comprising:

(a) culturing the genetically engineered host cell of claim 75 under conditions suitable to produce the polypeptide; and

(b) recovering the polypeptide from the cell culture.

77. An isolated polynucleotide comprising a polynucleotide that encodes a polypeptide fragment of SEQ ID NO:2, wherein said polypeptide fragment has endothelial cell proliferative activity.

78. The polynucleotide of claim 77 fused to a heterologous polynucleotide.

79. The polynucleotide of claim 77 fused to a polynucleotide which encodes a heterologous polypeptide.

80. A recombinant vector comprising the polynucleotide of claim 77.

81. A recombinant vector comprising the polynucleotide of claim 77 operatively associated with a regulatory sequence that controls gene expression.

82. A genetically engineered host cell comprising the polynucleotide of claim 77.

83. A genetically engineered host cell comprising the polynucleotide of claim 77 operatively associated with a heterologous regulatory sequence that controls gene expression.

84. A method for producing a VEGF-2 polypeptide, comprising:

(a) culturing the genetically engineered host cell of claim 83 under conditions suitable to produce the polypeptide; and

(b) recovering the polypeptide from the cell culture.

85. An isolated polynucleotide comprising a polynucleotide that encodes a polypeptide fragment encoded by the cDNA contained in ATCC Deposit No. 75968, wherein said fragment has endothelial cell proliferative activity.

86. The polynucleotide of claim 85 fused to a heterologous polynucleotide.

87. The polynucleotide of claim 85 fused to a polynucleotide which encodes a heterologous polypeptide.

88. A recombinant vector comprising the polynucleotide of claim 85.

89. A recombinant vector comprising the polynucleotide of claim 85 operatively associated with a regulatory sequence that controls gene expression.

90. A genetically engineered host cell comprising the polynucleotide of claim 85.

91. A genetically engineered host cell comprising the polynucleotide of claim 85 operatively associated with a heterologous regulatory sequence that controls gene expression.

92. A method for producing a VEGF-2 polypeptide, comprising:

(a) culturing the genetically engineered host cell of claim 91 under conditions suitable to produce the polypeptide; and

(b) recovering the polypeptide from the cell culture.

93. An isolated polynucleotide encoding a mature portion of a protein encoded by the cDNA contained in ATCC Deposit No. 75968.

94. The polynucleotide of claim 93 fused to a heterologous polynucleotide.

95. The polynucleotide of claim 93 fused to a polynucleotide which encodes a heterologous polypeptide.

96. A recombinant vector comprising the polynucleotide of claim 93.

97. A recombinant vector comprising the polynucleotide of claim 93 operatively associated with a regulatory sequence that controls gene expression.

98. A genetically engineered host cell comprising the polynucleotide of claim 93.

99. A genetically engineered host cell comprising the polynucleotide of claim 93 operatively associated with a heterologous regulatory sequence that controls gene expression.

100. A method for producing a VEGF-2 polypeptide, comprising:

(a) culturing the genetically engineered host cell of claim 99 under conditions suitable to produce the polypeptide; and

(b) recovering the polypeptide from the cell culture.

101. An isolated polynucleotide encoding a proprotein portion of a protein encoded by the cDNA contained in ATCC Deposit No. 75968.

102. The polynucleotide of claim 101 fused to a heterologous polynucleotide.

103. The polynucleotide of claim 101 fused to a polynucleotide which encodes a heterologous polypeptide.

104. A recombinant vector comprising the polynucleotide of claim 101.

105. A recombinant vector comprising the polynucleotide of claim 101 operatively associated with a regulatory sequence that controls gene expression.

106. A genetically engineered host cell comprising the polynucleotide of claim 101.

107. A genetically engineered host cell comprising the polynucleotide of claim 101 operatively associated with a heterologous regulatory sequence that controls gene expression.

108. A method for producing a VEGF-2 polypeptide, comprising:

(a) culturing the genetically engineered host cell of claim 107 under conditions suitable to produce the polypeptide; and

(b) recovering the polypeptide from the cell culture.

109. An isolated polynucleotide encoding a protein encoded by the cDNA contained in ATCC Deposit No. 75968.

110. The polynucleotide of claim 109 fused to a heterologous polynucleotide.

111. The polynucleotide of claim 109 fused to a polynucleotide which encodes a heterologous polypeptide.

112. A recombinant vector comprising the polynucleotide of claim 109.

113. A recombinant vector comprising the polynucleotide of claim 109 operatively associated with a regulatory sequence that controls gene expression.

114. A genetically engineered host cell comprising the polynucleotide of claim 109.

115. A genetically engineered host cell comprising the polynucleotide of claim 109 operatively associated with a heterologous regulatory sequence that controls gene expression.

116. A method for producing a VEGF-2 polypeptide, comprising:

(a) culturing the genetically engineered host cell of claim 115 under conditions suitable to produce the polypeptide; and

(b) recovering the polypeptide from the cell culture.

\* \* \* \* \*